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THE DETERMINATION OF NITROGEN BY MODIFIED KJELDAHL METHODS*

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It is well recognized that the Kjeldahl (1) method for nitrogen determination involves too great an expenditure of time, and numerous attempts have been made to shorten it. The introduction of catalysts or substances to raise the boiling point of the acid, such as the use of copper oxide and mercuric oxide advised by Wilfarth (2) and the addition of potassium or sodium sulfate by Gunning (3), or the later introduction of mixtures of sulfuric and phosphoric acids by Folin and Wright (4) or, still more recently, the combination of acid mixtures and potassium persulfate technique by Van Slyke (5), have contributed materially toward reducing the time necessary for digestion.

For some years we have used a mixture of equal volumes of water and concentrated sulfuric acid to which were added 1 gm. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 8 gm. of sodium sulfate per 100 cc. This mixture, made up in considerable quantities to save time, is reliable and effective in our hands. In an endeavor to save time, it was decided to investigate the properties of the vanadates, which have been employed in leather chemistry, as additional catalysts of the reaction. We had reached the opinion that sodium vanadate in amounts of 30 to 40 mg. would accelerate the reaction from 25 to 30 per cent, when the work on selenium came to our notice. Preliminary tests made it clear that much greater saving of time was possible with its use. In what follows we shall, therefore, discuss the latter catalyst.

Lauro (6) in 1931 proposed the use of selenium in the Kjeldahl reaction on the ground of equal accuracy, saving of time, and

* Read at the annual meeting of the Canadian Physiological Society at Kingston, Ontario, October 31, 1936.

reduction in cost. The subject has engaged the attention of the cereal chemists, and several papers (7-9) have appeared supporting his contentions. Recently, more critical papers by Davis and Wise (10) and Snider and Coleman (11) have appeared. The former state that the indications favor a lower result with its use and that its use with other common catalysts, especially mercury, is to be discouraged. Nevertheless they admit that it may prove quite satisfactory in the hands of some. Time of digestion, intensity of heat applied, and amount of sulfate used are factors which they list as important factors requiring investigation. It would appear significant that too much sulfate and too long a period of digestion, as previously noted by Sandstedt (9), influence the results unfavorably. Snider and Coleman show that, though lower temperatures prevail in the digests containing the selenium, the "critical point" occurs sooner, accompanied by a rise in temperature. Beyond this point there is a loss of nitrogen, probably due to concentration of the digest. Though results with mercury and selenium together as catalysts are unsatisfactory, selenium dioxide with copper appeared to work very well, results falling a little short with low protein-highly carbonaceous material. Further, the use of mossy zinc in combination with selenium causes frothing and may give rise to noxious fumes (hydrogen selenide) creating a danger to health.

The nitrogen determination of cereals is a more severe test for the method than those substances usually encountered in the clinical laboratory, but it should also be said that, in large part, the losses of nitrogen encountered by the above authors were probably those accompanying concentration of the acid, as the actual digestion must have been completed long before the arbitrary time allowed for digestion had elapsed. These criticisms, however, give emphasis to certain points one should avoid in the development of a method for nitrogen estimation with selenium as a catalyst.

Though numerous tests have been performed on other nitrogenous substances, such as urea, creatinine, blood serum, urine, corn-meal, etc., 0.6 gm. of a sample of grain curd casein, yielding 78.4 mg. of nitrogen, has been adopted as a standard of comparison for this work. The casein is digested with the stated amount of acid and the catalyst in question over a 600 watt enclosed

heater, and time is estimated from the moment of turning on the switch. Timing was sometimes arbitrary; at other times the digestion was continued until apparently complete. Distillation was carried out on rose gas burners. No antibumping agent and no antifothing mixture is required with the sulfuric-phosphoric acid mixtures. With the others, a few grains of acid- and alkali-washed quartz to prevent bumping and powdered pumice to control frothing during distillation were employed.

TABLE I
Digestion of 0.6 Gm. of Casein by Various Kjeldahl Methods

H ₂ SO ₄	CuSO ₄ ·5H ₂ O	K ₂ SO ₄	Selenium metal	Digestion time	Nitrogen yield	Remarks
cc.	mg.	gm.	mg.	min.	mg.	
25	0	0	0	125	78.4	Complete
25	200	0	0	35	77.9	Incomplete
25	0	0	100	22	78.0	"
25	200	0	100	20	77.8	"
20	300	10	100	12	78.4	Complete
20	500	10	100	11	77.4	Incomplete
20	600	10	100	11	78.1	"
20	300	10	100	12	78.0	"
		Na ₂ SO ₄				
20	0	0	0	13	77.1	300 mg. copper selenite used; in complete
20	400	0	0	20	77.0	0.7 gm. Hg; incomplete
15	150	2	0	50	78.4	Complete
15	150	2	100	18	78.0	Incomplete

Table I illustrates the digestion of 0.6 gm. of casein by various Kjeldahl methods. The original Kjeldahl procedure, sulfuric acid alone, gives a complete digestion in 125 minutes. The time is reduced to 50 minutes when 150 mg. of CuSO₄ + 2 gm. of Na₂SO₄ are used with three-fifths as much acid; and to 12 minutes when twice as much copper and 5 times as much sulfate are used together with 100 mg. of selenium metal. When other quantities of catalysts are used for short periods, the digestion is incomplete.

It seemed apparent that completion of the digestion in a decidedly shortened digestion period could be accomplished by

adding selenium to previously recognized catalysts. The process, however, is slow, as it involves weighing out or measuring the several ingredients. If possible, we would prefer the use of a single solution for digestion; and experiments were undertaken with this end in view. The 3:1 sulfuric-phosphoric acid mixture of Van Slyke was eventually adopted as a basis, and various catalysts were added. The substrate remained the same: 0.6 gm. of grain curd casein with a nitrogen content of 78.4 mg. As many of the substances to be analyzed contain water, in this series 10 cc. of water were added to each flask. 3 to 4 minutes are required to boil it off. As before, the time of digestion is measured from the moment of placing the flask on a cold 600 watt digester and turning the switch. Table II shows the results.

From the data presented in Table II it seems apparent that, with the 3:1 sulfuric-phosphoric acid mixture, considerable variation in the amount of copper and selenium gives a complete digestion of 0.6 gm. of casein in 10½ to 12 minutes. We have, therefore, incorporated in the sulfuric-phosphoric acid mixture 1 gm. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 1 gm. of SeO_2 ¹ per 100 cc. of acid. The two salts are suspended separately (each in half the acid) by means of heat, cooled somewhat, and poured together; then boiled. Some of the salt probably becomes copper selenate. On standing, a precipitate of copper salt may form. On boiling, this redissolves but, as exact proportions are not very important, the suspension may be used. With 20 cc. of this mixture, 0.6 gm. of casein is completely digested in 11 minutes; 99 per cent complete in 10 minutes; 98 per cent complete in 9 minutes; and 95 per cent complete in 8 minutes, when started in a cold 600 watt digester. Uniformly the reaction is complete when the mixture assumes a light blue color. 5 cc. of urine are digested in 7 to 10 minutes, depending on whether or not albumin or sugar is present. 1 cc. of serum requires about 7 minutes to complete the

¹ SeO_2 may be somewhat expensive and difficult to obtain. It is readily made from the powdered metal by heating gently with half concentrated nitric acid in a fume cupboard. Heating is continued until the substance is fully dry; a little water is added and evaporated off, taking the last traces of the nitric acid. None of the product is selenic trioxide, but on boiling in sulfuric acid it is more or less completely transformed from the lower to the higher oxidized form.

digestion. The time requirement is affected by the particular material to be analyzed, the volume of acid used, amount of heat applied, vigor of the suction fans, etc.

Some attention should be given to the size of the Kjeldahl flask used. The acid should cover fully the area to which heat

TABLE II

Digestion of 0.6 Gm. of Casein by 20 Cc. of Sulfuric and Phosphoric Acids, Various Catalysts Being Used

CuSO ₄ ·5H ₂ O	Sodium vanadate	Selenium metal	Time	Nitrogen yield	Remarks
mg.	mg.	mg.	min.	mg.	
Acid only	0	0	50	78.0	Incomplete
200	0	0	21	77.9	"
0	0	100	15	78.4	Complete
200	0	100	13	78.2	"
200	0	100	12	78.4	"
200	40	100	12	78.5	"
200	40	0	20	78.5	"
200	40	0	23	78.5	"
200	0	0	27	78.4	"
0	40	0	30	77.0	Incomplete
300	0	100	11	78.0	"
0	0	100	10	78.4	0.7 gm. Hg added; complete
0	0	0	11	78.4	0.3 gm. copper selenite added; complete
300	0	200	12	78.7	Complete
400	0	100	12	78.0	Incomplete
400	0	200	12	78.4	Complete
400	0	300	12	78.0	Incomplete
500	0	100	11	78.0	"
500	0	200	10½	78.0	"
600	0	100	10½	78.4	Complete
600	0	200	10½	78.4	"

is applied and heat leakage around the flask should be avoided to insure even boiling. A 300 cc. flask with 10 cc. of acid mixture is adequate for 5 cc. of urine or 1 cc. of serum when but a short period of digestion is required. 70 cc. of distillate will contain all the nitrogen. There is some danger of loss of nitrogen when mixing the alkali with the diluted acid mixture. The outflow

tube of the condenser should have a small opening or possibly a Folin bulb with numerous holes and be wider above to avoid sucking back of the standard acid, if the burner is affected by draughts.

The advantages claimed for this single digestion mixture are the saving in time over the other methods tried and the definiteness of the end-point of digestion. Potassium sulfate and mercury, which were shown to give unsatisfactory results with selenium, have been replaced by phosphoric acid. A clear, light blue color (which does not change until concentration of the digest occurs) marks the end-point of digestion. We are aware that this may not be the case when other nitrogenous substances are digested but, so far, this difficulty has not been encountered.² Iron, instead of copper, may be used along with selenium with equally good results, but the definiteness of the end-point is lacking. In our opinion there is no advantage in the separate addition of copper selenite dihydrate as recently recommended by Schwoegler, Babler, and Hurd (12).

Five flasks containing 0.6 gm. of casein digested with 20 cc. of the mixture for arbitrary periods gave the following results: 11 minutes, 78.3 mg. of nitrogen; 12 minutes, 78.4 mg.; 15 minutes, 78.4 mg.; 20 minutes, 78.6 mg.; 30 minutes, 78.3 mg.; average, 78.4 mg. of nitrogen.

As to the cost, the most expensive ingredient in the Kjeldahl estimation of nitrogen has been the time of the analyst. A slightly increased cost for chemicals is balanced by a diminished cost for heat, while the saving of time is very considerable.

Microdetermination of Nitrogen—While microdeterminations of nitrogen are now less necessary from the standpoint of economy of time, the need for them still exists when but a limited amount of material is available for analysis. The copper-selenium-acid mixture may be used for this purpose, preferably diluted 1:2 with water to reduce the viscosity. It is also quite suitable for the estimation of the non-protein nitrogen of tungstic acid filtrates or trichloroacetic acid filtrates of blood. As Van Slyke

² With some organic chemicals, where great difficulty of digestion is encountered, we would suggest that, to compensate for loss of selenium and acid, successive portions of the warmed digestion mixture be added to the cooling flask each 30 minutes until digestion is complete.

(5) has previously noted, etching of the glassware is almost entirely avoided by using a smaller proportion of phosphoric acid than is used in the Folin-Wu (13) acid digestion mixture. The flame must not be allowed to touch the glass uncovered by acid.

The digestion technique is the same as that of Folin and Wu and the time required is approximately the same. The nitrogen in the digestion mixture may be determined by steam distillation and titration (Pregl's technique) or by colorimetric comparison after direct nesslerization. If the colorimetric estimation be preferred, some care should be taken to boil the acid quite vigorously at the end of digestion in order to wash down and redissolve a red precipitate of selenium which appears on the cold part of the tube during the early stages of the digestion. When 0.5 cc. of acid is used (1 cc. of diluted acid), 15 cc. of Nessler's solution (Folin-Wu formula) are required to develop full color in a 50 cc. volume; 20 cc. in a 100 cc. volume. The standard is similarly prepared simultaneously with the unknown.

SUMMARY

When selenium is added to a 3:1 mixture of sulfuric and phosphoric acids containing copper, a rapid and effective digesting agent for digesting nitrogenous matter is produced. Suggestions as to its use are included in the paper.

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SULFITES AS PROTEIN PRECIPITANTS*

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One of the earliest and most important of the methods for differentiation and characterization of proteins was their precipitation from colloidal solution by inorganic salts. The beginnings of this study date back almost a century and the progress made has been adequately reviewed on several occasions by Schryver (1), Robertson (2), Handovsky (3), Cohn (4), and others. It may therefore suffice to say here that a very considerable amount of investigation has been carried out, showing the effect on various proteins of numerous inorganic salts. It is curious, however, that the effects of the sulfites on proteins appear to have been overlooked, so far as we have been able to discover. Some experience with sulfites in another connection led us to suspect them of a capacity for salting-out proteins, and it is of interest to record briefly some of the data obtained. They present a number of new possibilities in the fractionation of the proteins, which will require much further work for their complete elucidation.

Doubtless, as is the case with other salts, the particular proteins used for testing will influence the result. In this work human blood serum or plasma was used in a dilution of 1 part of serum to 19 parts of the saturated aqueous solution of the particular sulfite, acting for 10 minutes at a room temperature of 20° unless otherwise noted. Where precipitation of the proteins is incomplete, there is a tendency in some cases for the process to continue if allowed to act for a longer time, but this rule is subject to certain exceptions. Likewise, more complete precipitation is attained by raising the temperature of the solutions to

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TABLE I
Effect of Saturated Solutions of Various Sulfites on Serum and Plasma at Room Temperature

Saturated sulfite solution	Solubility	Serum			Oxalated plasma			Remarks
		Opalescence	Precipitate	Redissolves on dilution	Opalescence	Precipitate	Redissolves on dilution	
Aluminum.....	Slight	+	Slight	-		++	-	Permanently insoluble
Ammonium.....	+++		+++	+		+++	+	Complete precipitation
" bisulfite.....	+++		+++	+		+++	+	"
Barium.....	+++		+++	-	+	-	-	Clots
Calcium.....	Slight	+	-	-		++		Permanently insoluble
Lead.....	"	+	++	-		+++		Globulin precipitation incomplete
Lithium.....	++		+	+		++	+	
Magnesium.....	+	+	-			+	-	Becomes insoluble
Potassium.....	+		+	+		+	+	Fibrin precipitation incomplete
" metabisulfite.....	++		+++	Immediately		+++	Partly	Becomes insoluble rapidly
Sodium.....	+++		+++	+		+++	+	Albumin precipitation incomplete
" bisulfite.....	+++		+++	+		+++	+	Becomes complete and partly insoluble in 24 hrs.
Strontium.....	Slight	+	-	-	+	-	-	Complete; permanently insoluble
Zinc.....	+		+++	-		+++		

37° by using solutions supersaturated at room temperature, or by employing solutions fully saturated at 37° at this temperature. In many instances the protein salted-out is redissolved by the dilution of the salt solution with an equal or sometimes, as in the case of plasma, twice its volume of water. In other instances the salt action becomes irreversible with greater or less rapidity.

Table I summarizes roughly the effect of a number of sulfites on serum and plasma respectively at room temperature in comparison to their solubility. The sign + + + + + indicates complete salting-out of the plasma proteins, while + + + + in the serum column also indicates maximal precipitation. An opalescence may be observed on the addition of certain sulfites to serum or plasma. In some instances this opalescence becomes more marked with considerable rapidity and a precipitate appears within an hour—frequently, much more rapidly. In other instances the opalescence remains unchanged or gives way to slight precipitation in the 24 hour period. Solutions saturated at a higher temperature often increase the completeness of the process. In the case of certain sulfites individual comment seems desirable.

Comment

Ammonium sulfite, likewise the bisulfite, precipitates all the protein on full saturation of the solution. As with the ammonium sulfates, precipitation of certain protein fractions can be produced with undersaturated solutions. With serum, potassium sulfite causes marked opalescence and very slight precipitation within the hour at room temperature. More protein is precipitated with saturated solution at 40°. The suspension filters quickly, but clear filtrates are difficult to obtain. Precipitation of fibrinogen is incomplete at room temperature. Potassium metabisulfite is unstable. The rapidly flocculent precipitate of part of the globulins, which filters off readily, is soluble on dilution, but the process later becomes irreversible. With this salt, and sodium bisulfite as well, considerable frothing is experienced during the distillation of ammonia nitrogen by the Kjeldahl method.

Lithium sulfite is moderately soluble and precipitates considerable protein. At room temperature the globulin precipita-

tion is incomplete, however. The precipitate redissolves on dilution. Though flocculating well, the filtration is somewhat slow. The filtrate bumps tremendously during distillation of the ammonia nitrogen. Magnesium sulfite is relatively slightly soluble and produces only slight opalescence with serum even at 40°. With plasma a partial precipitation of fibrinogen takes place, which soon fails to redissolve on dilution. Saturated solutions of lead, aluminum, and zinc sulfites produce precipitates of globulins which rapidly become irreversible. Some albumin is also precipitated by the zinc sulfite. The sulfites of calcium, strontium, and barium are rather too insoluble to be of much interest in this connection. They merely produce opalescence or minor degrees of precipitation with serum. If the oxalate concentration is sufficiently low, calcium sulfite causes clotting of the fibrinogen of oxalated blood plasma. On heparinized plasma only an opalescence is produced.

Sodium bisulfite in saturated solution salts-out the serum globulins as a rather gelatinous mass, slow to filter, resembling in this respect the salt precipitation with magnesium sulfate. Precipitation of the albumins is incomplete at room temperature. The normal sodium sulfite in saturated solution removes the globulins and part of the albumin rapidly from solution. The precipitate separates well. There is an early tendency to flocculate and filtration is rapid. Filtrates made after 10 minutes remain clear. Precipitation of the albumins is still incomplete, however, when saturated solutions at 40° are employed.

It will be noted that, in general, the precipitating power of the sulfites for the protein of human serum or plasma is limited largely by the solubility of the particular salt employed. This is equivalent to saying in the words of Howe (5), "for a given anion the cation is the determining factor in precipitation" (without prejudice to the effect of various anions having a common cation). Increased solubility due to increased temperature improves the precipitating power of the sulfite.

Examination of Table I indicates that some of the sulfites may have practical value either alone or in combination with other salts in such procedures as require salting-out processes. Some of these possibilities are still under investigation. The succeeding article discusses one of these in more detail: the determination

of albumin, globulin, and fibrinogen in blood serum and plasma by means of sulfites. The most useful sulfites for this purpose would seem to be those of ammonium, lithium, sodium, and zinc. Of these the first has obvious disadvantages when the estimation requires a nitrogen estimation, though protein partitions can be made with it. The zinc salt causes irreversible precipitation which continues to completion if sufficient zinc is present relative to the total protein concentration. Lithium sulfite in saturated solution (approximately 19.5 per cent) precipitates fibrinogen and euglobulin without the pseudoglobulins or albumin. Even though, in half saturated solution and over, a few flakes of protein may be precipitated, which fail to redissolve on dilution, there are possibilities of using it in appropriate concentrations as a reagent for fibrinogen and euglobulin. Our greatest objection to it is the bumping and sputtering in the Kjeldahl nitrogen estimation. The use of bisulfite of sodium as a precipitant for salting-out protein is somewhat unsatisfactory on account of the unstable nature of the salt. Comparatively fresh solutions are advisable. Of these precipitants, the remaining one—normal sodium salt—is possessed of the most valuable properties.

At room temperature, saturated sodium sulfite solution contains about 28 per cent of the anhydrous salt; at 40° approximately 30 gm. per 100 cc. are held in solution. The saturated solution is relatively stable chemically and will stand cooling about 4° before precipitation commences. Also it will return readily to solution at 4° above room temperature. The 21 per cent solution (used in the following paper) should not be kept in the refrigerator, as precipitation will occur, but the salt remains in solution at 15°.

The suggestion of Howe, that salts insufficiently soluble to effect a precipitation individually may be used in combination, finds some support in the results of saturating a solution of potassium sulfite with sodium sulfite. The former, at room temperature, is soluble to the extent of 15.3 per cent, in other words 0.75 mole, of the hydrated salt $K_2SO_3 \cdot 2H_2O$, and just fails to precipitate fibrinogen which is precipitated by 0.75 mole of sodium sulfate. Sodium sulfite in saturated solution (2.0+ moles per liter) fails to precipitate all the albumin, which Howe has shown can be precipitated completely by a 2.75 M concentration of sodium

sulfate. The mixture of saturated sodium sulfite in saturated potassium sulfite causes complete precipitation of serum protein. Saturation of a saturated solution of sodium sulfite with sodium bisulfite is ineffective in this regard, as is also saturation of a saturated solution of lithium sulfite with sodium sulfite. In the latter instance only half the amount of sodium sulfite required to saturate water will dissolve.

The possible importance of these salts in the separation and concentration of certain sera is under investigation. The use of sodium sulfite as a protein precipitant for separation of albumin and globulin in blood is described in the following article. Also, in certain clinical cases some apparent abnormalities of the kind and amount of the globulins occur, a circumstance which has aroused our interest.

SUMMARY

Many sulfites in suitable concentration will precipitate proteins. The properties of a number of these have been investigated and described. While certain other sulfites would be useful for salting-out certain protein fractions, the normal sodium sulfite is the most satisfactory for use with the proteins of blood plasma.

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THE ALBUMIN, GLOBULINS, AND FIBRINOGEN OF SERUM AND PLASMA*

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Determination of Albumin and Globulin

Various procedures have been employed to determine the amount of albumin and globulin in a serum or plasma. As one of the earliest distinctions between the two groups of proteins rests on the fact that globulins are precipitated by half saturation with ammonium sulfate, whereas albumins are only salted-out by complete saturation of the mixture with the salt, this procedure furnishes a basis for many of the methods. It is now certain that, however satisfactory it may be as a simple dividing line between the two groups of proteins, the differentiation is not specific and other salting-out processes are quite as useful, though an exact parallelism between the results obtained and the values obtained by one of the ammonium sulfate methods is not achieved.

Cullen and Van Slyke (1) have devised a method of determination of globulin and albumin dependent upon half saturation of the plasma with ammonium sulfate, removal of the ammonium sulfate by distillation with magnesium oxide, digestion of the residue, and distillation of the ammonia derived from protein. This valuable method suffers from the disadvantage of requiring the preliminary removal of the ammonium sulfate before determining the nitrogen from albumin, and the severe bumping encountered in its removal—circumstances which induced Howe (2) to develop his method of using sodium sulfate as the globulin precipitant. The considerable advantage of this later technique

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has led Peters and Van Slyke (3) to recommend it, while certain disadvantages have led to the devising of several variant procedures by others. The method in its best form requires a 12 hour period in the hot room or incubator at 37° to effect a salt precipitation of the globulins, together with filtration at the same temperature. 3 hours standing at 37° suffice at times, but this is not invariably true with abnormal sera and doubtless accounts for some of the bizarre results reported. Several years ago in a preliminary communication we (4) described a method of accelerating the process by the addition of talc. The variability in the adsorptive qualities of different specimens of talc makes this device inapplicable except after a careful study of the talc employed, and we have turned our attention in other directions for a rapid but accurate method.

With normal sera, refractometry by Robertson's (5) method gives reproducible results, but the results are quite in error when the particular serum is abnormally constituted. An alternative method based upon estimation of the tyrosine content of the protein was published by Wu (6) in 1922, and further modified by Wu and Ling (7), Greenberg (8), Looney (9), Cameron *et al.* (10), and others. Our experience with these methods has perhaps been too limited to assess them properly. Peters and Van Slyke, however, suggest in a foot-note to their discussion caution in accepting unreservedly values obtained in cases of nephrosis by methods based upon the tyrosine content of proteins.

None of the methods involving separation of proteins by salting-out methods and determination of the nitrogen content of the precipitate takes account of the fact that the nitrogen-containing lipids are carried down in the precipitate. This error is usually quite small, and, if important, could probably be reduced or avoided by introduction of an extraction with a lipid solvent in the technique. It is, of course, eliminated when filtrate protein is determined, but remains as a source of error in the estimation of the total protein and, therefore, increases the true value of those fractions determined by difference. It is of less importance than the conventional adoption of $N \times 6.25$ as the standard mode of calculating the proteins of blood plasma.

In connection with the newer methods of nitrogen determination which permit a great reduction in time required for its

completion, it seemed opportune to inquire whether the Howe method might also be improved with respect to the time factor by substituting the use of sulfites for sulfates in separation of the proteins. In the preceding paper (11) the general characters of the salting-out process by various sulfites are recorded. It seems apparent that sodium sulfite presents the greatest possibilities in this direction.

Howe (2) has shown that dilutions of the serum or plasma with 10 to 30 times its volume of sodium sulfate solution give satisfactory precipitations. Selection of a ratio of 1 part of serum to 19 parts of sulfite solution was made for ease of calculation of the result, because it provides a volume of filtrate which can be measured with considerable accuracy and because as compared to a 1:9 dilution the filtration is much faster. The results with a 1:9 dilution, however, check well within the limits of experimental error with the 1:19 dilution.

Howe also found 22.2 per cent sodium sulfate solution, a concentration which can only be kept in solution in the hot room or incubator at 37°, necessary to accomplish the complete salting-out of the globulins. This method, in which the 12 hour period for precipitation is used, served us as a standard with which to compare the results of precipitation at room temperature in a 1:19 dilution of sera by varying concentrations of the sodium sulfite solution. Concentrated solutions of sodium sulfite at room temperature remove not only all the globulins but part of the albumins from solution. Since it was readily shown that no further precipitation with sodium sulfite took place after standing 10 minutes, this was adopted as a standard minimum time for precipitation. No error is introduced by increasing this period to suit the convenience of the analyst. The results shown in Table I are calculated to show the amount of protein per 100 cc. of serum remaining in the filtrate after precipitation of the globulin fraction. It will be noted that over a range of 4 per cent variation in concentration of the sodium sulfite solution, *viz.* 20, 21, 22, and 23 per cent, constant values are obtained for the protein remaining in solution and that, moreover, experimental error discounted, these values correspond accurately with the values for serum albumin by the Howe technique. With 19 per cent and with 24 per cent sodium sulfite solutions the results

for filtrate protein frequently correspond with these values, but this has not been a constant finding. We have, therefore, adopted an intermediate figure, 21 per cent, for the concentration of sodium sulfite solution in this method, which may be described briefly as follows:

Method

1 part of serum accurately measured is diluted with 19 parts of a 21 per cent solution of sodium sulfite and mixed by repeated inversion. After standing 9 minutes, 1 drop of caprylic alcohol¹ is

TABLE I

Effect of Various Concentrations of Sodium Sulfite Solutions on Filtrate Protein (Albumin) Concentration

The values are given in gm. per cent.

Serum of subject.	B	C	D	H	L	Mc	A	G	Do	S	F	M	Gw
Total protein....	7.88	7.10	6.85	7.52	7.60	7.75	6.94	7.70	7.90	7.30	7.1	4.00	7.10
Albumin, Howe method	5.08	4.52	4.36	4.70	5.40	5.50	4.44	5.00	3.30	5.30	4.50	2.40	4.80
Na ₂ SO ₃ concentration													
20%.....	5.00	4.70	4.24	4.74	5.44	5.56	4.44	5.00	3.40	5.16	4.56	2.44	4.80
21%.....	5.10	4.60	4.32	4.78	5.46	5.60	4.44	5.04	3.40	5.20	4.52	2.44	4.80
22%.....	5.00	4.60	4.30	4.74	5.46	5.60	4.40	5.04	3.36	5.16	4.50	2.44	4.76
23%.....	4.80	4.56	4.24	4.70	5.36	5.40	4.20	4.96	3.10	5.00	4.40	2.36	4.60
25%.....	4.32	4.12	3.68	4.12	4.80	5.20	4.00	4.80	2.80	4.80	4.10	2.00	4.40
Saturated..				3.30			3.40	3.80	2.10		3.60		

added; the mixture is shaken and stands 1 minute. It is then filtered through a No. 40 Whatman filter paper. The first 1 cc. of filtrate is discarded or poured back into the filter paper. 10 cc. of filtrate (equivalent to 0.5 cc. of original serum—less may be used if necessary) are digested with the copper-selenium-phosphoric-sulfuric acid mixture previously described (12) with the precautions as noted. The nitrogen, as ammonia, is distilled and caught in 10 cc. of N/17.5 acid and titrated back with N/17.5 alkali. Titration is carried out with a corrected automatic 10 cc. burette graduated in 0.02 cc., with a small stainless steel needle

¹ The caprylic alcohol is not necessary but improves somewhat the aggregation of the globulins.

TABLE II

Serum Protein Values (in Gm. Per Cent). Na_2SO_3 Technique Compared with Howe Method

Case	Total protein	Howe albumin	Na_2SO_3 albumin	Difference	Howe globulin	Na_2SO_3 globulin	Diagnosis
	7.10	4.60	4.70	0.10	2.50	2.40	Mixed serum
M. E.	4.85	2.65	2.86	0.21	2.20	1.99	2nd stage glomerulonephritis
C. A.	5.60	2.95	3.05	0.10	2.65	2.55	Acute nephritis
B. O.	5.52	3.83	3.94	0.11	1.69	1.58	" "
M. A.	7.70	5.45	5.60	0.15	2.25	2.10	Malignant hypertension
H. F.	6.50	2.73	2.73	0.00	3.77	3.77	Multiple myeloma
"	5.92	2.78	2.90	0.12	3.14	3.02	" "
L. K.	6.80	4.02	4.10	0.08	2.78	2.70	Amyloid disease; tuberculosis
H. N.	7.33	5.14	5.32	0.18	2.19	2.01	Coronary thrombosis
R. S.	6.69	4.55	4.60	0.05	2.14	2.09	" "
H. A.	6.80	4.72	4.66	-0.06	2.08	2.14	Myocarditis; hypertension
D. G.	6.69	4.21	4.16	-0.05	2.48	2.53	" auricular fibrillation
Z. I.	7.20	4.10	4.28	0.18	3.10	2.92	Arthritis; pregnancy
W. G.	7.00	3.92	4.00	0.08	3.08	3.00	Infected finger
I. G.	8.10	4.30	4.40	0.10	3.80	3.70	" hand
H. T.	7.52	4.70	4.78	0.08	2.82	2.74	Anxiety neurosis
L. I.	7.60	5.40	5.46	0.06	2.20	2.14	Disseminated sclerosis
McN.	7.75	5.50	5.60	0.10	2.25	2.15	Progressive muscular atrophy
K. K.	8.00	4.10	4.24	0.14	3.90	3.76	Amyloidosis; tuberculosis of hip
K. R.	7.40	3.90	4.00	0.10	3.50	3.40	" " " ver- tebræ
A. R.	5.96	3.90	4.00	0.10	2.06	1.96	" "
W. N.	6.96	5.10	5.20	0.10	1.86	1.76	" "
K. G.	6.80	4.68	4.80	0.12	2.12	2.00	Amyloidosis; tuberculosis of hip
R. E.	6.36	2.70	2.60	-0.10	3.66	3.76	" pulmonary tubercu- losis
P. E.	8.08	4.04	4.10	0.06	4.04	3.98	Amyloidosis; tuberculosis of ver- tebræ
B. T.	7.88	5.08	5.10	0.02	2.80	2.78	Epilepsy
C. O.	7.10	4.52	4.60	0.08	2.58	2.50	Hypertension

TABLE II—*Concluded*

Case	Total protein	Hove albumin	Na ₂ SO ₄ albumin	Difference	Hove globulin	Na ₂ SO ₄ globulin	Diagnosis
H. E.	6 85	4 36	4 32	-0 04	2 49	2 53	Hemiplegia
A. G.	6 94	4 40	4 44	0 04	2 54	2 50	Infective asthma
G. N.	7 70	5 00	5 04	0 04	2 70	2 66	Lobar pneumonia
L. D.	7 90	3 30	3 40	0 10	4 60	4 50	Chronic degenerative myocarditis; hypertension; lues
S. R.	7 30	5 30	5 20	-0 10	2 00	2 10	Chronic tonsillitis
W. H.	5 40	3 50	3 60	0 10	1 90	1 80	Hyperthyroidism
M. N.	5 00	3 30	3 26	-0 04	1 70	1 74	Bilateral renal cortical necrosis
T. E.	8 00	5 30	5 40	0 10	2 70	2 60	Thrombophlebitis of renal veins
C. Y.	7 76	5 00	5 10	0 10	2 76	2 66	Arthritis
V. C.	7 25	4 85	4 84	-0 01	2 40	2 41	"
S. S.	5 40	2 70	2 72	0 02	2 70	2 68	Tuberculosis
N. V.	7 55	4 54	4 52	-0 02	3 01	3 03	"
M. E.	4 60	2 40	2 44	0 04	2 20	2 16	Glomerulonephritis, 2nd stage
G. W.	7 10	4 80	4 80	0 00	2 30	2 30	Addison's disease
L. O.	4 20	2 17	2 24	0 07	2 03	1 96	Hepatic cirrhosis
C. R.	5 20	3 44	3 44	0 00	1 76	1 76	Diabetes; hypothyroidism
M. A.	7 60	5 40	5 40	0 00	2 20	2 20	Malignant hypertension
K. L.	6 30	3 50	3 60	0 10	2 80	2 70	Portal cirrhosis
C. R.	5 80	4 35	4 30	-0 05	1 45	1 50	Diabetes; hypothyroidism
H. E.	5 00	3 42	3 52	0 10	1 58	1 48	Pernicious anemia
S. N.	6 75	4 80	4 90	0 10	1 95	1 85	Multiple myeloma
C. A.	6 40	3 75	3 70	-0 05	2 65	2 70	Acute nephritis
S. N.	6 45	4 40	4 44	0 04	2 05	2 01	Multiple myeloma
C. K.	6 80	3 90	3 90	0 00	2 90	2 90	Primary carcinoma of liver
S. D.	6 25	3 40	3 44	0 04	2 85	2 81	Carcinoma of stomach
G. R.	6 30	3 70	3 76	0 06	2 60	2 54	Acute nephritis
D. S.	5 30	3 50	3 56	0.06	1 80	1 74	Cholecystitis

tip dropping 92 drops per cc. It is convenient to use chemicals with zero or negligible nitrogen blank and to make the normality of the solutions exact. Then 10 minus the burette reading is the

albumin in gm. per 100 cc. of serum. When but 5 cc. of filtrate are used, the figure obtained is, of course, doubled. The globulin value is obtained by subtracting the albumin value from the total protein value obtained by Kjeldahl determination of the nitrogen content. When 0.5 cc. of serum is used, the same calculation applies.

The non-protein nitrogen as a source of error has been neglected to this point. 16 mg. of non-protein nitrogen per 100 cc. of serum would cause an error of 0.1 gm. of protein per 100 cc. When the non-protein nitrogen of the blood is known to be abnormal, the suitable amount should be subtracted from both the total protein value and the albumin value of the serum. When normal, no significant error will be introduced by subtracting 0.2 gm. (equals 32 mg. of non-protein nitrogen) per 100 cc. from the total protein and albumin values obtained above.

Table II provides a comparison of albumin and globulin values of serum obtained from hospital patients by the standard Howe technique with those obtained by the use of a 21 per cent sodium sulfite solution as the globulin precipitant. The first eighteen sera show a somewhat greater difference between the values obtained by the two methods than do the later determinations. They were analyzed before the introduction into the method of the caprylic alcohol and second shaking. It may be pointed out, however, that the differences are less than those which may occur by changing the sulfite concentration (see Table I) or the sulfate concentration used by 1 per cent (2). In any case, these differences are negligible from a clinical point of view and fall too close to the limits of experimental error to be regarded as essential differences between the two methods. It seems apparent then that the results attained with sodium sulfite solution show a material decrease in the time required and difficulties inherent in Howe's original method and furnish a solution, from a clinical standpoint, for a rapid method of separation of the albumin and globulin of serum.

Determination of Fibrinogen in Oxalated and Heparinized Plasma

In methods for the separation of fibrinogen or fibrin generally use is made of the procedure developed by Cullen and Van Slyke (1), on oxalated blood or citrated blood (Gram (13)), of recalcify-

ing a diluted plasma and separating the fibrin formed. Alternatively, precipitation of the fibrinogen by means of salts has been used by Howe (14). For the estimation of the protein, the determination of its nitrogen content by a macro- or micro-Kjeldahl technique (1), freeing from extraneous substances and drying to constant weight (13, 15), and colorimetric determination of a constituent, such as tyrosine (6-10), have been used. Howe (14) has used the estimation of the nitrogen content of a plasma filtrate following a recalcification or a salting-out procedure. The difference between the nitrogen value obtained and the nitrogen of the original plasma represents the nitrogen of the fibrin or fibrinogen. Of these methods the most convenient and reliable in our hands has been the Cullen and Van Slyke procedure—conversion of the fibrinogen of diluted plasma to fibrin by recalcification and estimation of the fibrin nitrogen by macro-Kjeldahl technique. The latter process is accelerated by the use of the copper-selenium-phosphoric-sulfuric acid mixture previously described (12). We prefer, however, to allow at least 3 hours at room temperature for the completion of the coagulation process which, with the necessary washing procedure, makes the process somewhat lengthy. When other tests are required on the same patient, 5 cc. of plasma are frequently not available for this test. With experience in the method one may, it is true, fall back on the micro form of this method with good results, provided sufficient care is exercised, but the numerous manipulations necessary open more sources of error when the smaller quantity of plasma is used.

In their present form, the usual methods also are best carried out with oxalated or citrated plasma. The increasing use of heparinized plasma which possesses the advantage of disturbing few of the normal relationships of the blood is difficult to fit in with these methods, since comparatively slight excess of heparin causes marked inhibition of fibrin formation.

The sharp separation of sodium sulfite precipitates of fibrinogen and their marked tendency to aggregate made it seem possible that a simple method might be devised for its determination. It was first necessary to make certain that no other protein in blood would simulate fibrinogen under the conditions of the test. In a series of sodium sulfite solutions increasing in concentration

by 0.5 per cent and a dilution ratio of solution to *plasma* of 19:1, the first evidences of precipitation occur with 9.5 per cent sodium sulfite solution. At room temperature the opalescence first seen increases with time, but precipitation is still incomplete in 24 hours. With successively higher concentrations the precipitate increases in density and rapidity of precipitation and filters out readily in 10 minutes with 12 and 12.5 per cent concentrations of the salt. Increasing the temperature to 37° causes a better aggregation of the particles, however, and has been incorporated in the method given below for this reason. Examination of some hundreds of *sera* in a similar fashion shows that, with the exception of a haze in the solution due to large amounts of red blood cells, to a fat meal, or to marked hemolysis, few *sera* show any opalescence after 10 minutes standing diluted with 13.5 per cent sodium sulfite solution; fewer still show opalescence with 13 per cent; and but one with 12.5 per cent solution. The opalescence seen with the 13 per cent concentration of salt passes through filter paper after the material has stood for 10 minutes, but a slight amount of protein is precipitated out after it has stood for 24 hours. Most of the *sera* in which this has occurred are from jaundiced patients, though not all *sera* from jaundiced patients exhibit it. All the other *sera* become opalescent with 13 per cent sodium sulfite solution after standing 24 hours, but do not precipitate. The single instance in this series in which a 12.5 per cent solution of sodium sulfite caused precipitation in a serum was from a case of multiple myelomata with amyloid tumors. The peculiar protein in the serum is not Bence-Jones protein nor, in our opinion, is it a euglobulin. A very slight precipitate was caused by a 12 per cent sodium sulfite solution.

Method

In a clean 50 cc. Pyrex centrifuge tube with a short cone bottom² place exactly 0.5 cc. of oxalated or heparinized plasma. Add 9.5 cc. of 12.5 per cent sodium sulfite solution, mix, and stand the tube in a water bath at 37° for 10 minutes. (It is unnecessary that a temperature of 37° be maintained throughout

² The centrifuge tube sold by Arthur H. Thomas Company under catalogue No. 3124-A or 3124-B is satisfactory. The long cone type creates difficulties in the micro-Kjeldahl procedure.

this period; standing in a beaker of water whose initial temperature does not exceed 40° is quite satisfactory.) A white precipitate separates out and aggregates into larger masses. Centrifuge at 2500 R.P.M. for 5 minutes. The precipitate collects in the bottom of the tube in a compact mass, leaving the supernatant fluid quite clear. Pour off the supernatant fluid and drain the tube 1 minute, wiping off the last drop clinging to the mouth of the tube. Break up the precipitate by spurting in about 5 cc. of the sodium sulfite solution and shaking; recentrifuge; pour off, and drain. Add 0.5 cc. of the acid mixture (12) and two quartz pebbles. Digest over a microburner, covering with a watch-glass when dense white fumes appear in the tube. Digestion is complete in 2 minutes. Allow to cool 3 minutes and add approximately 2 cc. of water. At this point a choice of methods for estimation is available: steam distillation and titration of the ammonia formed, or nesslerization. We have been agreeably surprised to find that the latter process has checked very satisfactorily with the Cullen-Van Slyke values by a macro-Kjeldahl technique. Probably, when the other technique is not in constant use, it represents the method of choice, as it is considerably faster. Transfer the liquid quantitatively to a 100 cc. graduate or volumetric flask, washing out the tube with successive portions of water until the total volume approximates 70 cc. Add 20 cc. of Nessler's solution (Folin-Wu formula), make to volume, and mix at once. Compare in a colorimeter with a standard containing 0.40 mg. of nitrogen as ammonia similarly treated with the plunger set at 20 mm. $(20/R) \times 0.4 \times 200 \times 6.25 = \text{fibrinogen, mg. per cent.}$ $(10/R) = \text{fibrinogen, gm. per cent.}$

In Table III will be found a comparison of the fibrinogen values obtained by both 12 and 12.5 per cent Na_2SO_3 with the fibrin values of the same blood obtained by the Cullen-Van Slyke procedure. It will be seen that the values obtained by using 12.5 per cent Na_2SO_3 compare very satisfactorily with the Cullen-Van Slyke procedure for fibrin, though a difference of 10 per cent has been encountered in one case. We are inclined to attribute this to the use of excess oxalate to prevent coagulation, but shortage of material prevented its repetition. Even in this case, however, the clinical value of the test would not be impaired. A greater amount of variability is to be noted when the results obtained

TABLE III
Comparison of Plasma Fibrinogen Determinations (Na_2SO_3 Procedure) with
Fibrin by Cullen-Van Slyke Method
The values are given in gm. per cent.

Plasma No.	Cullen-Van Slyke fibrin	Fibrinogen, using Na_2SO_3		Diagnosis
		12.5 per cent	12.0 per cent	
1	0.395	0.400	0.395	Diabetes mellitus
2	0.330	0.337	0.339	" "
3	0.466	0.475	0.470	" "
4	0.400	0.407	0.412	" "
5	0.332	0.333	0.323	" "
6	0.444	0.445	0.440	Jaundice
7	0.820	0.815	0.800	Glomerulonephritis
8	0.225	0.252	0.238	Jaundice
9	0.390	0.400	0.403	"
10	0.430	0.411	0.400	Multiple myeloma
11	0.236	0.245	0.242	Gastric carcinoma
12	0.466	0.476	0.487	Diabetes mellitus
13	0.490	0.500	0.496	Jaundice
14	0.700	0.705	0.715	Arthritis
15	0.484	0.476	0.470	Gastric ulcer
16	0.400	0.392	0.390	Arthritis
17	0.225	0.237	0.245	Disseminated sclerosis
18	0.450	0.435	0.415	Peripheral neuritis
19	0.500	0.470	0.480	Acute gastroenteritis
20	0.410	0.417	0.414	Pernicious anemia
21	0.475	0.488	0.495	Hodgkin's disease
22	0.444	0.455	0.465	Cerebral thrombosis
23	0.365	0.377	0.385	Pernicious anemia
24	0.245	0.250	0.227	Herpes zoster
25	Heparinized	0.703	0.690	Silicosis; asthma
26	plasma used,	0.427	0.424	Auricular tachycardia
27	Cullen-Van	0.520	0.500	Chronic degenerative myocarditis
28	Slyke proce-	0.455	0.445	Spondylitis
29	dure impossi-	0.292	0.290	Diabetes; gastric carcinoma
30	ble	0.318	0.312	" mellitus
31		0.550*	0.561	" "
32		0.555*	0.561	" "
33		0.525*	0.535	" "
34		0.510*	0.500	" "
35		0.670	0.650	Gastric ulcer
36		0.615	0.608	Normal

* Oxalated plasma taken at the same time gave values 6 to 11 per cent lower.

with 12 per cent Na_2SO_3 are compared with the others. As a rule slightly less protein is precipitated, but this is not invariable.

In a short series, using heparinized plasma, we obtained a distinctly better correlation between the results obtained with 12.5 and 12 per cent sodium sulfite. This we are inclined to attribute to the absence of the disturbing effect of the oxalate. Unfortunately, fibrin estimations cannot be carried out on heparinized bloods; thus direct comparisons are impossible. In four instances marked above portions of the same bloods were carefully oxalated and heparinized respectively. The results checked well, though the oxalated bloods showed values 6 to 11 per cent lower both for fibrin and fibrinogen, as is also the case with similar comparisons of albumin and globulin content. Probably this is attributable to plasma dilution due to the oxalate. While the dilution effect could perhaps be kept constant by adding definite quantities of blood to known quantities of oxalate and separating the plasma and the cells at a definite time after mixing, in actual practise sufficient care is seldom taken of this factor. The use of a heparinized plasma is, therefore, more satisfactory in that the dilution factor is non-existent; the analysis, however, must be performed within 24 hours. To provide for contingencies we employ about 5 times the theoretical requirement of standardized heparin measured in solution and allowed to dry in the test-tube at room temperature. The values given are not averages of duplicates but a group of consecutive single determinations made in parallel with 12.5 per cent and 12 per cent sodium sulfite solutions as the precipitants. Though a somewhat more severe test, the maximum difference between the two determinations is but 4 per cent. With oxalated blood we would recommend that precautions be taken with regard to the amount of oxalate used relative to blood and the time of separation of the plasma and that determinations be made in parallel with 12.5 per cent sodium sulfite solution as the precipitant. As the determinations of the fibrinogen content of the plasma are based on analyses of the precipitated protein, no allowance for the non-protein nitrogen of the plasma is required.

Determination of Globulin Fractions

Though similar observations had been made previously by Porges and Spiro (16) with the same salt, Howe (2) has shown

most completely that with sodium sulfate solutions four "zones of precipitation" occur on treating a plasma-protein mixture with increasing concentrations of the salt. These zones of precipitation correspond with a considerable degree of accuracy to the fibrinogen content, the euglobulin content, and the total globulin content of the plasma, as determined by other methods, and indicate a difference between a pseudoglobulin I and a pseudoglobulin II. It becomes, then, of some interest to determine whether or not sodium sulfite behaves in a similar fashion to sodium sulfate. In Table IV are collected some data bearing on this point. Dilution of the plasma or serum is made in a ratio of 1:19 with solutions of sodium sulfite varying in concentration by 0.5 per cent. The actual concentration of sodium sulfite in the mixture in gm. per 100 cc. will be 19/20 of the concentration of the sulfite solution.

Examination of Table IV will show that zones of precipitation corresponding to the globulin fractions now recognized are obtained by the use of sodium sulfite instead of sodium sulfate as the protein precipitant. This corroborative evidence, it should be stated, does not necessarily indicate that these fractions are specific chemical entities. It is not improbable that some overlapping occurs and that the concentrations most suitable for precipitating the fractions from a mixture such as plasma are not necessarily those to which the isolated protein would respond most advantageously. It has also been shown by Howe that the sodium sulfate precipitation does not yield quite the same amount of globulin as when the conventional ammonium sulfate precipitation is used, and it is probable that this is also true with sodium sulfite as the precipitant. It is of interest to note a close correlation between the values obtained on serum and heparinized plasma.

It has previously been shown that precipitation of the fibrinogen is complete with 12 per cent or 12.5 per cent of sodium sulfite solution (see previous section). The euglobulin fraction would appear approximately the same in amount when 14.5 per cent or 15 per cent or 15.5 per cent sodium sulfite solutions are used. The middle value (15 per cent) has been chosen as representing a reasonably accurate separation. In the three instances examined it agrees satisfactorily with the results of the sodium chloride precipitation—the classical though somewhat troublesome

TABLE
*Filtrate Protein Determinations on Heparinized Plasma and Serum with Various Concentrations of Sodium Sulfite**
 The values are given in gm. per cent.

Case	Plasma or serum	Total protein	Percentage of sodium sulfite solution employed												Remarks
			13.5	14	14.5	15	15.5	16	16.5	17	17.5	18	18.5	21	
M	Serum	7.12	7.70	7.12	6.60	6.65	6.20	6.13	5.80	5.70	5.30	5.25	5.15	4.70	Pooled sera
H	"	7.70	7.70	7.60	7.20	7.10	7.12	6.80	6.76	6.48	6.20	6.08	6.00	5.70	
F	"	6.80	6.80	6.60	6.30	6.14	6.10	5.56	5.52	5.56	5.52	5.50	5.40	5.16	
C	Heparinized plasma	6.6	6.60	6.40	6.20	6.16	5.90	5.66	5.54	5.52	5.50	5.48	5.30	5.04	Fibrinogen 0.731%
	Serum	8.30	7.60	6.70	6.40	6.36	6.20	5.90	5.84	5.80	5.60	5.40	5.32	4.84	
D	Heparinized plasma	7.80	6.80	6.56	6.36	6.24	6.08	5.88	5.88	5.88	5.88	5.56	5.40	4.90	
	Serum	7.70	7.00	6.60	6.32	6.00	5.80	5.68	5.60	5.60	5.52	5.48	5.20	4.88	Fibrinogen 0.67%
Cn	Heparinized plasma	6.96	6.68	6.40	6.24	6.04	5.80	5.76	5.72	5.72	5.60	5.56	5.36	4.96	
	Serum	6.34	5.72	5.52	5.40	5.30	5.10	5.00	4.84	4.80	4.56	4.52	4.36	4.04	
S	Heparinized plasma	7.76	5.56	5.40	5.32	5.20	5.00	4.92	4.80	4.60	4.56	4.46	4.48	4.00	Fibrinogen 0.615%
	Serum	6.08	5.60	5.40	5.32	5.20	5.00	4.88	4.80	4.60	4.56	4.46	4.32	4.10	
B	"	5.70	5.60	5.40	5.30	5.10	4.96	4.84	4.72	4.56	4.56	4.32	4.16		
	Heparinized plasma	6.84	6.60	6.40	6.16	6.04	5.80	5.60	5.50	5.40	5.20	5.16	5.16	4.60	Fibrinogen 0.425%
	Serum	7.70	7.10	7.00	6.76	6.70	6.60	6.40	6.20	6.16	6.24	5.92	5.84	5.56	
Bl	Serum	7.20	7.16	7.00	6.80	6.72	6.56	6.48	6.36	6.04	6.08	6.00	5.88	5.50	

* It has already been shown above that a 12.5 per cent sodium sulfite solution is suitable for fibrinogen estimation and that the zone of precipitation of total globulins extends from 19 to 24 per cent sodium sulfite solution and is best represented by a 21 per cent solution. As it would appear to be unnecessary to increase the number of confirmatory instances for these, the values for filtrate protein with a 12.5 per cent sodium sulfite solution are omitted and only those for a 21 per cent sodium sulfite solution are used. After removal of fibrinogen, save in a few rare and decidedly abnormal instances, no precipitation occurs with a 13 per cent sodium sulfite solution; these figures also are, therefore, omitted.

method for separating euglobulin from serum. Inspection of the data of Table IV also shows a marked tendency for the 17.5 per cent and 18 per cent sulfite solutions to precipitate an equal quantity of globulin. Quite frequently the 18.5 per cent solution salts-out a closely similar amount and less frequently the 17 per cent solution removes from solution about the same quantity of protein as the 17.5 and 18 per cent solutions. It may be concluded, then, that the 18 per cent sodium sulfite solution is the more suitable concentration to use in separating out the pseudoglobulin I fraction. It has already been shown that precipitation of all the globulins is very satisfactorily accomplished by the use of 21 per cent sodium sulfite solution. The additional globulin salted-out by this concentration is known as pseudoglobulin II.

It may be recalled that Howe (17) found that, with sodium sulfate solution, salting-out of the globulin fractions was best accomplished by adding 0.25 mole to the molar strength of the next more dilute member of the series. Thus, fibrinogen requires sodium sulfate solution of 0.75 M strength to salt-out the protein, euglobulin 1.0 mole, etc. With lithium sulfate the addition of 0.4 gm. molecule per liter was required to accomplish the same result. With other salts a different increment of salt is required. Having regard for the fact that the actual concentration of salt in the mixture is 19/20 of the concentration of the sulfite solution employed, instead of 12, 15, 18, and 21 per cent solutions, we are actually dealing with 11.4, 14.25, 17.1, and 19.95 per cent of sulfites in the mixture, or 0.906, 1.131, 1.355, and 1.58 M concentrations, an arithmetical progression increasing by 0.225 mole. While the existence of definite zones of precipitation is perhaps insufficient in itself to warrant the division of the globulins into four groups of proteins, there are certain other evidences pointing in this direction. Among these are the absence of the euglobulin and pseudoglobulin I fraction, discovered by Howe (18), from the blood of new born calves, and its appearance following the first feeding of the colostrum which contains proteins having a similar zone of precipitation, the specific binding properties for amylose possessed by the euglobulin fraction (19), and the marked variation in the amount and kind of the globulin fractions in the blood of patients suffering from several disorders. Incidentally, with sulfites as the precipitant, the absence of the euglobulin

fraction and the pseudoglobulin I fraction from the serum of new born calves is not paralleled in man. In the examples of human placental blood shown in Table V the presence of these fractions was demonstrated.

TABLE V
Globulin Fractions in Placental Blood Serum Precipitated by Sulfites
The values are given in gm. per cent.

Case No.	Na ₂ SO ₃			Total globulin	Total albumin	Total protein
	15 per cent	18 per cent	21 per cent			
1	0.52	0.60	0.28	1.40	4.52	5.92
2	1.20	1.60	0.40	3.20	4.40	7.60
3	1.08	0.76	0.52	2.36	5.64	8.00
4	0.70	0.60	0.30	1.60	4.20	5.80
5	0.90	0.46	0.44	1.80	4.20	6.00

TABLE VI
Parallel Determinations of Globulin Fractions (Sulfite Method)
The values are given in per cent; the globulin values are set in bold-faced type.

	Case 1		Case 2	
	a	b	a	b
Total protein.....	7.3	7.3	7.0	7.0
15% sulfite filtrate.....	6.1	6.16	5.28	5.36
Euglobulin.....	1.2	1.14	1.72	1.64
18% sulfite filtrate.....	4.80	4.76	4.40	4.44
Pseudoglobulin I.....	1.3	1.40	0.88	0.82
21% sulfite filtrate.....	4.08	4.04	3.84	3.84
Pseudoglobulin II.....	0.72	0.72	0.56	0.60
Total globulin by difference.....	3.22	3.26	3.16	3.16
“ “ “ addition.....	3.22	3.26	3.16	3.08

For several reasons direct determination of the precipitated globulin fractions is not at present satisfactory except in the case of fibrinogen. Since the determination of these globulins is dependent upon a difference in value between the albumin or albumin plus globulin content of the serum and the total protein of the serum, the accumulated error falls on the particular globulin fraction. The reproducibility of fractionation results in

parallel experiments on the same sample of blood is reasonably satisfactory as in the following examples of serum from two cases of Hodgkin's disease recently encountered (see Table VI).

TABLE VII

Concentration of Globulin Fractions in Human Blood

The values are given in per cent.

Case	Serum or plasma	Fibrinogen	Euglobulin	Pseudo-globulin I	Pseudo-globulin II	Total globulin
	Serum		*0.47	1.40	0.55	2.42
M	"		0.60	1.02	0.38	2.00
H	"		0.66	0.62	0.36	1.64
F	"		0.44	0.68	0.44	1.56
C	Heparinized plasma	0.731	1.24	0.96	0.56	2.76
	Serum		1.44	0.80	0.66	2.90
D	Heparinized plasma	0.67	1.00	0.52	0.60	2.12
	Serum		0.72	0.68	0.60	2.00
Cn	Heparinized plasma	0.615	0.42	0.78	0.48	1.68
	Serum		0.44	0.76	0.56	1.76
S	Heparinized plasma	0.425	0.40	0.74	0.36	1.50
	Serum		0.40	0.74	0.40	1.54
B	"		0.68	1.00	0.56	2.24
Bl	Heparinized plasma	0.615	0.40	0.78	0.36	1.54
	Serum		0.44	0.72	0.50	1.66
Fr	Heparinized plasma	0.60	0.80	0.32	0.52	1.64
I	" "	0.60	0.50	0.70	0.40	1.60
Su	" "	0.40	0.90	0.40	0.70	2.00
Ho	" "	0.60	0.80	0.60	0.40	1.80
T	" "	0.40	1.20	0.48	0.32	2.00
A	Oxalated "	0.24	0.56	0.40	0.40	1.36
V	Serum		0.24	0.56	0.96	1.76
Sn	"		0.65	0.80	0.36	1.81
Br	"		0.59	0.44	0.32	1.35
L	"		0.80	1.20	0.84	2.84
G	"		0.70	1.10	0.80	2.60
W	"		0.48	0.60	0.50	1.58
Br	"		0.90	1.22	0.40	2.52

In each case the "b" determinations were made independently 48 hours after the "a" determinations. A certain amount of coincidence and benefit of averages is probably present in these determinations. Nevertheless, from the individual variations shown and the results in Table IV it would seem that it is unde-

sirable to trust the results of fractionation to more than 0.1 gm. per 100 cc. of serum. A similar conclusion is reached on determining fibrinogen by difference with a 12.5 per cent sodium sulfite precipitation in comparison with the direct determination as previously described. In five instances fibrinogens by difference were 0.6, 0.6, 0.4, 0.6, and 0.4 gm. per 100 cc., while the direct fibrinogen determinations were 0.572, 0.588, 0.445, 0.582, and 0.455 gm. per 100 cc. respectively.

With the limitations mentioned above, then, the various globulin fractions may be determined by the use of 12.5 per cent, 15 per cent, 18 per cent, and 21 per cent sodium sulfite solutions as the precipitants and it may be of interest to assemble the data in Table IV together with later determinations, using only the concentrations of sodium sulfite selected above in order to illustrate the approximate range of concentration of these fractions in human blood (hospital patients). The results will be found in Table VII. Though it is known that abnormalities in these concentrations occur in multiple myeloma and lymphadenoma, there is little information at hand regarding other conditions which, we feel, will repay further study. Some work, not as yet complete, would seem to indicate that, while specific separations are not possible in a single precipitation, the separation is somewhat more sharply defined with sulfites than with sulfates and this may have some importance in the separation and concentration of antitoxic sera, etc.

SUMMARY

Determination of the albumin and globulin content of serum can be accomplished simply and rapidly by the use of sodium sulfite as the protein precipitant and a copper-selenium-phosphoric-sulfuric acid mixture as the protein digestant. The results obtained by the technique described are identical with those obtained by Howe's technique with sodium sulfate.

With a 12.5 per cent sodium sulfite solution, fibrinogen may be rapidly salted-out of plasma whether oxalated, citrated, or heparinized. When collected by centrifugation and with the protein estimated by determination of its nitrogen content, the values compare well with those of the longer Cullen-Van Slyke macroprocedure.

When sodium sulfite solutions of varying concentrations are employed, it is possible to show that the globulin fractions of serum or plasma present zones of maximal precipitation of protein, if the concentration of the solution is changed by approximately 0.225 mole. These fractions parallel the fibrinogen, euglobulin, pseudoglobulin I, and pseudoglobulin II content of the plasma, as previously found by Porges and Spiro, Howe, and others.

The euglobulin and pseudoglobulin I fractions are represented in human placental blood.

The accuracy of the partition of the serum proteins is discussed. A series of determinations on the blood of hospital patients is recorded showing the probable range of concentration of the various fractions. It seems possible that information of clinical value may be obtained from a further study of the globulin fractions.

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ON PROTEOLYTIC ENZYMES

XV. REGARDING THE GENERAL NATURE OF INTRACELLULAR PROTEOLYTIC ENZYMES

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Papain

Papain has been shown to contain two partial enzymes (1) which will be designated in this paper as Papain I¹ and Papain II. Papain I is strongly inhibited by phenylhydrazine, while Papain II is not affected by this reagent. In the action of papain-HCN on gelatin at pH 5, both Papain I and Papain II participate in the proteolysis. If the same enzymatic hydrolysis is carried out in the presence of a sufficient quantity of phenylhydrazine, the splitting of gelatin is performed only by Papain II. The course of gelatin hydrolysis by papain-HCN in the presence or absence of phenylhydrazine is presented in Fig. 1.

It will be noted from Fig. 1 that Papain II is capable of carrying out an extensive degradation of gelatin even without the concurrent action of Papain I. Papain II hydrolyzes about one-half of all the peptide linkages.² The great extent of proteolysis by papain supports the view that the action of the proteinases is not confined to high molecular substrates.

The finding of the dual enzyme nature of papain suggested the hypothesis (1, 2) that the unactivated papain was a combination of the two partial enzymes and that the process of activation

¹ The term Papain I is employed to replace the previously proposed Papain Peptidase I.

² The splitting of all the peptide linkages would correspond to 12 mg. of NH₂-N per cc.

involved a dissociation into two enzyme components. A series of experiments was performed to test this theory.

Papain preparations which have been freed as much as possible from the naturally occurring phytokinase by repeated treatment with hydrogen sulfide and alcohol (3) split gelatin quite rapidly to a moderate extent, and after a short interval no further change is

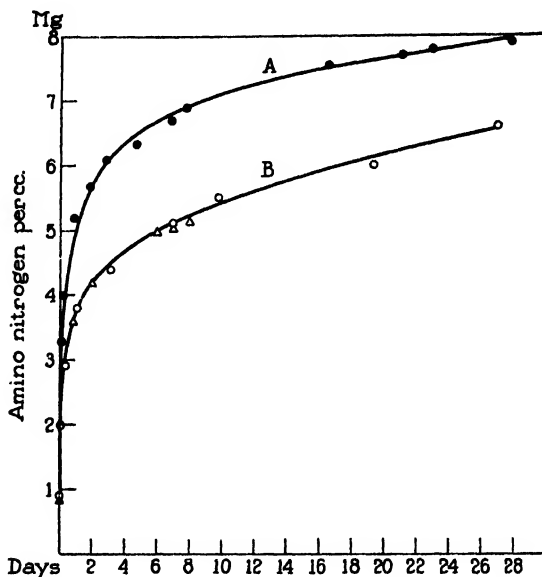


FIG. 1. Hydrolysis of gelatin by papain-HCN in the presence of phenylhydrazine. Curve A, 4 gm. of commercial papain + 120 gm. of Atlantic gelatin + 3 gm. of potassium cyanide + 250 cc. of citrate buffer of pH 5 + 750 cc. of water; Curve B, C as in Curve A + 10 gm. of phenylhydrazine, Δ 2.5 gm. of purified papain + 120 gm. of Atlantic gelatin + 3 gm. of potassium cyanide + 10 gm. of phenylhydrazine + 250 cc. of citrate buffer of pH 5.0 + 750 cc. of water. Temperature 40°.

observed (Fig. 2, Curve A). If to an enzyme preparation of this kind phenylhydrazine is added and is followed after some time by gelatin, there occurs a rapid and extensive hydrolysis of the protein (Fig. 2, Curve B). In a third parallel experiment the enzyme was first treated with phenylhydrazine to inactivate Papain I, and after several hours an amount of benzaldehyde was added which was equivalent to the phenylhydrazine. 2

hours afterwards gelatin was added and the resulting hydrolysis of the protein was very slight (Fig. 2, Curve C).

These experiments indicate that if Papain I is removed from the gelatin-papain system by means of phenylhydrazine, Papain II is automatically activated. The subsequent addition of benzaldehyde regenerates Papain I, but there results an inactive enzyme solution, not a mixture of active Papain I and active Papain II. The return of Papain I into the solution containing Papain II thus produces an extensive inactivation of the two partial en-

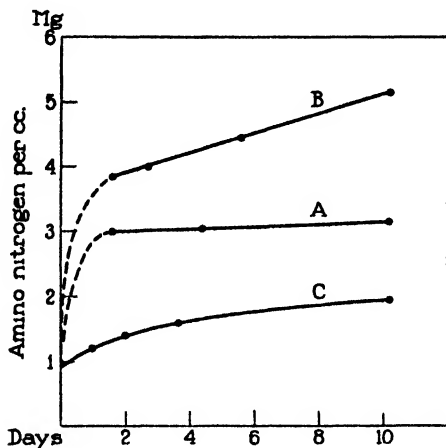
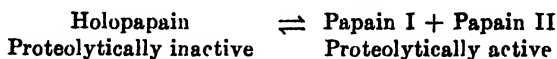


FIG. 2. Hydrolysis of gelatin by purified papain. Curve A, 0.4 gm. of purified papain + 12 gm. of Atlantic gelatin + 25 cc. of citrate buffer of pH 5.0 + 75 cc. of water; Curve B, as in Curve A + 1.5 gm. of phenylhydrazine; Curve C, as in Curve B + 1.5 gm. of benzaldehyde.

zymes. The two partial enzymes, when they exist in the same relative proportions as in the original papain preparation, inactivate each other so completely that only a slight initial splitting of gelatin can occur.

It was recently shown that with papain-HCN the results of such experiments are different (1). In this case there are active Papain I and active Papain II at the start. Addition of phenylhydrazine inactivates Papain I, and an equivalent amount of benzaldehyde regenerates this enzyme component; but because of the presence of HCN, Papain I and Papain II cannot inactivate each other.

In some of the recent literature (4) the activation of papain has been interpreted as a reduction process, and the reversible inactivation of the enzyme as an oxidation. In our opinion, this interpretation does not adequately explain the experiments with phenylhydrazine in which one of the partial enzymes is activated under the same conditions as the other partial enzyme is inactivated. The results of these experiments suggest strongly that the activation is to be considered as a dissociation in the following sense.



(Holopapain is the designation used for the compound of Papain I with Papain II.)

The question may arise whether, in addition to the dissociation, a reduction process plays some significant rôle in the activation. For Papain II it may be shown that after activation by phenylhydrazine the addition of HCN produces no additional activation. In Fig. 3 the action of papain-phenylhydrazine and papain-phenylhydrazine-HCN is compared. It will be noted that the presence of HCN had only a slight inhibitory effect.

It was reported (1) that Papain I, after inactivation by phenylhydrazine, cannot be reactivated by the addition of HCN. In Table I there are presented two experiments in which Papain I was first inactivated by means of different quantities of phenylhydrazine, then treated with HCN, and finally allowed to act upon carbobenzoxyisoglutamine at 40°. After 22 hours there was no noticeable splitting. However, the addition of cysteine or glutathione to Papain I-phenylhydrazine yields entirely different results. These sulfhydryl compounds are able to regenerate the activity of Papain I. It is possible that the reactivation by means of sulfhydryl compounds depends on a partial dissociation of the combination between Papain I and phenylhydrazine.

The activation of papain was first described by Mendel and Blood (5) who noted a more far reaching proteolysis of gelatin and other proteins by papain upon the addition of HCN or H₂S. The action of these activators was believed by Willstätter and Grassmann (6) to consist in an extension of the specificity range of the enzyme. Following the discovery of a natural activator

which was present also in the commercial preparations of the enzyme, Grassmann and Dyckerhoff (7) expressed the view that

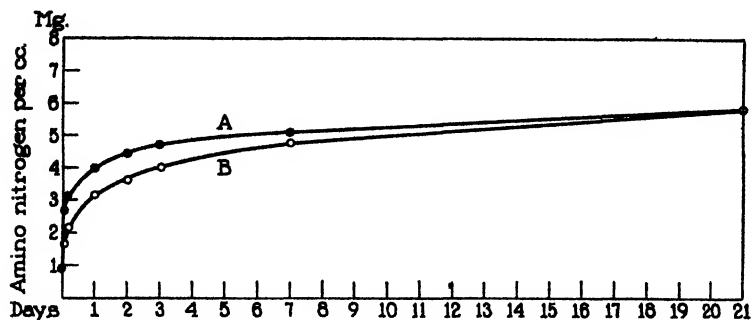


FIG. 3. Hydrolysis of gelatin by papain-phenylhydrazine in the presence of HCN. Curve A, 4 gm. of papain + 120 gm. of Atlantic gelatin + 10 gm. of phenylhydrazine + 250 cc. of citrate buffer of pH 5; Curve B, as in Curve A + 3 gm. of potassium cyanide.

TABLE I

Hydrolysis of Carbobenzoxyisoglutamine by Papain I

The splitting was measured in cc. of 0.01 N KOH per 0.2 cc. of test solution. 1 cc. increase represents 100 per cent splitting for one peptide linkage.

Reagent	Reagent per cc. solution	Hydrolysis		
		1 hr.	3 hrs.	22 hrs.
HCN.....	0.018	0.47		0.96
".....	0.018			
C ₆ H ₅ NHNH ₂	0.02			
HCN.....	0.018			0.01
C ₆ H ₅ NHNH ₂	0.05			
Cysteine.....	0.005			
".....	0.005	0.25	0.55	0.76
C ₆ H ₅ NHNH ₂	0.05			
Glutathione.....	0.005			
".....	0.005	0.36	0.55	0.74
C ₆ H ₅ NHNH ₂	0.05			
".....	0.005	0.14	0.17	0.39
C ₆ H ₅ NHNH ₂	0.05			

papain itself is proteolytically inactive but may be activated by different activators to perform different tasks. The limited ex-

tent of gelatin splitting by natural papain was interpreted by Maschmann and Helmert (8) as depending on the catalytic oxidation of the natural —SH activator by traces of copper in the gelatin. In view of these contradictory theories it seemed desirable to reinvestigate the effect of non-activated papain on gelatin.

In Fig. 4 there is reproduced the course of the digestion of a low ash gelatin preparation (Eastman Kodak) by a papain preparation which had been freed as completely as possible from the natural activators. The protein is split at the start but the proteolysis

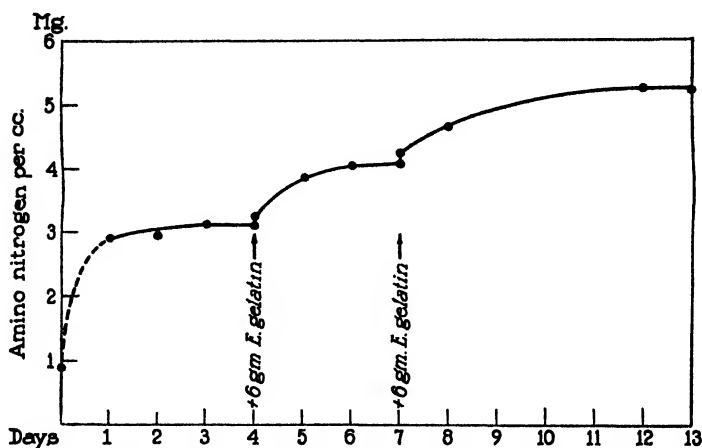


FIG. 4. Effect of addition of gelatin on papain hydrolysis. 0.25 gm. of purified papain + 12 gm. of Eastman gelatin + 25 cc. of citrate buffer of pH 5 + 75 cc. of water.

soon comes to a standstill. The addition of fresh gelatin produces a new brief digestion; a third addition of protein shows the same result. This experiment indicates that in the absence of activators papain attacks gelatin and its first degradation products but is inactive toward the smaller degradation products. A possible explanation for this behavior may be that gelatin and its first degradation products have so great an affinity to one or both of the partial enzymes of papain that they force a dissociation of the holopapain into the component enzymes. This high affinity would probably be lacking in the smaller degradation products; these cannot prevent the reformation of holopapain.

The foregoing experiments indicate the necessity of distinguishing between the substrates which are split by the enzyme without the assistance of activators and the substrates which require such activators. The enzyme which is inactive toward one substrate may be active toward another substrate. The expressions "active enzyme" and "inactive enzyme" therefore lead to an ambiguity in the case of papain.

Synthetic Substrates for Liver Cathepsin and Bromelin

In animal cells and organs there occur proteinases which may be activated by hydrocyanic acid and sulfhydryl compounds. These enzymes are designated cathepsins and are differentiated according to source as liver cathepsin, spleen cathepsin, etc. In order to compare the cathepsins of various normal and pathological tissues it is necessary to obtain simple substrates of known structure. It was possible to find that cathepsin from hog liver splits the following substrates: carbobenzoxydiglycylglycine, carbobenzoxy-*L*-leucylglycylglycine and its amide, carbobenzoxyglycyl-*L*-glutamylglycineamide, and carbobenzoxyglycyl-*L*-tyrosylglycineamide. The last named substrate is split at two peptide linkages. It is of interest, in regard to enzyme specificity, that carbobenzoxy-leucylglycylglycine is split more rapidly than the corresponding amide. Diglycyl-*L*-leucylglycine and triglycyl-*L*-leucylglycine are split by liver cathepsin with remarkable ease (Table II).

It will be noted from Table III that carbobenzoxy-leucylglycylglycine is split by cathepsin in the presence of cysteine to 100 per cent in 17 hours. The same substrate is scarcely attacked following the addition of phenylhydrazine. The originally active enzyme is thus inhibited by phenylhydrazine. In a third experiment, benzaldehyde was added following phenylhydrazine and then the action of the enzyme toward the substrate was regenerated. The enzyme which splits carbobenzoxy-leucylglycylglycine and is inhibited by phenylhydrazine may be designated Cathepsin I or, more precisely, Liver Cathepsin I. Cathepsin I behaves toward phenylhydrazine in the presence of activators in quite the same manner as does Papain I.

The cathepsin preparation employed effected no noticeable hydrolysis of albumin peptone within 5 hours. However, upon

addition of phenylhydrazine a strong hydrolysis resulted. A further experiment in which benzaldehyde had been added besides the phenylhydrazine showed only a slight hydrolysis. The com-

TABLE II

Behavior of Synthetic Substrates toward Cathepsin and Bromelin

The splitting was measured in cc. of 0.01 N KOH per 0.2 cc. of test solution. 1 cc. increase represents 100 per cent splitting for one peptide bond in the synthetic substrates.

Substrate	Cathepsin		Bromelin	
	Time	Hydrolysis	Time	Hydrolysis
	Ara.		Ara.	
Hippurylamide	21	0.01	23	-0.02
Benzoyl-L-isoglutamine	10	-0.02	23	0.01
	24	0.00		
Chloroacetyl-L-tyrosine	20	0.00		
	46	-0.01		
Carbobenzoxdiglycylglycine	10	0.28	23	0.01
	24	0.42		
Carbobenzox-L-leucylglycylglycine	20	0.84		
Carbobenzox-L-leucylglycylglycineamide	26	0.26		
	49	0.50		
Carbobenzoxglycyl-L-glutamylglycineamide	20	0.23	20	0.78
	46	0.35	44	0.96
			68	1.05
Carbobenzoxglycyl-L-isoglutamine			21	0.06
Carbobenzoxglycyl-L-tyrosylglycineamide	26	0.50	29	1.45
	49	1.05	53	1.96
	72	1.37		
Benzoyl-L-leucyl-L-leucylglycine			21	0.04
Carbobenzoxdiglycyl-L-leucylglycine			21	0.01
Diglycyl-L-leucylglycine	21	0.97	21	-0.01
Triglycyl-L-leucylglycine	21	1.12	21	-0.01
Diglycyl-L-glutamylglycine	21	0.35	21	-0.02
Gelatin	4	0.58	2	0.87
	10	1.06		

ponent of liver cathepsin, which is not inhibited by phenylhydrazine and has properties similar to those of Papain II, may be designated Cathepsin II or Liver Cathepsin II.

On the basis of the same considerations as in the case of papain,

liver cathepsin may therefore be regarded as a dual enzyme, and its activation as a dissociation process. Both of the partial

TABLE III

Splitting by Cathepsin and Bromelin in Presence of Various Reagents

The hydrolysis is measured in cc. of 0.01 N KOH per 0.2 cc. of solution.

Substrate	Reagent	Reagent per cc. solution	Cathepsin		Bromelin	
			5 hrs.	17 hrs.	2 hrs.	30 hrs.
Albumin peptone		<i>mM</i>	0.02		0.10	0.33
	C ₆ H ₅ NHNH ₂	0.04	0.48		0.12	0.59
	"	0.04				
Gelatin	C ₆ H ₅ CHO	0.04	0.09			
			0.35	0.95	0.42	1.11
	C ₆ H ₅ NHNH ₂	0.04	0.32	0.90	0.43	1.07
	Cysteine	0.03	0.52	2.13		
	"	0.03				
	C ₆ H ₅ NHNH ₂	0.04	0.42	0.95		
	Cysteine	0.03				
	C ₆ H ₅ NHNH ₂	0.04	0.54	1.48		
	C ₆ H ₅ CHO	0.04				
	HCN	0.05			0.77	1.71
	"	0.05				
	C ₆ H ₅ NHNH ₂	0.04			0.35	0.83
	HCN	0.05				
	C ₆ H ₅ NHNH ₂	0.04			0.57	1.54
	C ₆ H ₅ CHO	0.04				
Carbobenzoxy-l-leu- cylglycylglycine	Cysteine	0.03		1.00		
	"	0.03				
	C ₆ H ₅ NHNH ₂	0.01		0.05		
	Cysteine	0.03				
	C ₆ H ₅ NHNH ₂	0.01		0.91		
Carbobenzoxyglycyl- l-glutamylglycine- amide	C ₆ H ₅ CHO	0.01				
	HCN	0.05				0.78
	"	0.05				
	C ₆ H ₅ NHNH ₂	0.01				0.01
	HCN	0.05				
	C ₆ H ₅ NHNH ₂	0.01				0.52
	C ₆ H ₅ CHO	0.01				

enzymes participate in the splitting of gelatin in the presence of cysteine. Addition of phenylhydrazine inhibits Cathepsin I so that the initial splitting of gelatin is slowed down.

In 1930 Waldschmidt-Leitz *et al.* (9) reported the existence of a catheptic enzyme in hog liver, which, when activated, effected a slow splitting of chloroacetyltyrosine, benzoyl- and phthalylglycylglycine, carbethoxyglycylleucine, and perhaps leucylglycyltyrosine. This enzyme was considered to be a catheptic carboxypeptidase and its separation from cathepsin was described. Our crude enzyme preparation was completely inactive toward chloroacetyltyrosine under conditions in which carbobenzoxy-leucylglycylglycine was split to 84 per cent. Furthermore, our enzyme hydrolyzed carbobenzoxy-leucylglycylglycineamide and carbobenzoxyglycyltyrosylglycineamide which contain no carboxyl and therefore cannot be attacked by a carboxypeptidase. The enzyme belongs rather to the group of endopeptidases (proteinases).

The assumption of a special catheptic carboxypeptidase was required at a time when proteinases were not believed to be capable of attacking substrates of low molecular weight such as benzoylglycylglycine. Since this view has been found to be incorrect in numerous instances, there is insufficient basis at present to assume the existence of a catheptic carboxypeptidase.

It was found that the enzyme of the pineapple, bromelin, in the presence of hydrocyanic acid splits the following substrates: carbobenzoxyglycyl-*L*-glutamylglycineamide and carbobenzoxyglycyl-*L*-tyrosylglycineamide, the latter at two linkages (Table II). The splitting of carbobenzoxyglycylglutamylglycineamide is strongly inhibited by phenylhydrazine but is regenerated by benzaldehyde (Table III). The enzyme responsible for this behavior may be called Bromelin I. If in bromelin-HCN the Bromelin I is inhibited by phenylhydrazine, there still remains another active enzyme which effects an appreciable splitting of gelatin. This enzyme may be called Bromelin II.

The bromelin preparation employed showed an appreciable splitting of albumin peptone even without the addition of activators. However, an addition of phenylhydrazine produced a greater rate of splitting. Thus, the relationship between the two component enzymes of bromelin is essentially similar to the case of papain or cathepsin.

The question has been raised in the literature regarding the number of different papainases. It has been suggested that all

the plant papainases are identical to the enzyme from *Carica papaya* and, in particular, that papain and bromelin are identical enzymes (10). It was possible to test this view by means of the synthetic substrates. A comparison of the data presented in Table I for Cathepsin I and Bromelin I with previously published results for Papain I shows great specificity differences among these three enzymes. For example, activated Cathepsin I does not attack benzoylisoglutamine in contrast to Papain I. Furthermore, activated bromelin is inactive toward a series of peptides and peptide derivatives which are split by papain.

EXPERIMENTAL

The preparation of the synthetic substrates employed in this paper has been described in previous publications of this series.

The commercial papain preparation (1) was purified by precipitation with alcohol according to Grassmann (3). It was found that 2.5 gm. of the purified papain were equivalent in enzymic potency to 4 gm. of the commercial preparation. Cathepsin was prepared by desiccating pig liver with alcohol, extracting the dry material with 87 per cent glycerol, and filtering (9). The bromelin was prepared from pineapple juice by ammonium sulfate precipitation (10).

Cathepsin was activated by cysteine just before use for hydrolysis; bromelin was incubated with HCN for 2 hours at 40° for activation. In the phenylhydrazine experiments the reagent was left in contact with the enzyme solution for 2 hours before testing. A similar interval followed the addition of benzaldehyde; the benzaldehyde phenylhydrazone was centrifuged off, and the clear supernatant liquid was employed.

The concentration of the synthetic substrates was in all cases 0.05 mm per cc.; the concentration of peptone and gelatin was 47 mg. per cc. and 40 mg. per cc. respectively, except where otherwise stated. The amounts of the enzymes used in the experiments with synthetic substrates were as follows: cathepsin, 0.5 cc. of the glycerol extract per cc. of test solution; bromelin, 4 mg. of the dry preparation per cc.; papain, 2.25 mg. of the dry preparation per cc. Cathepsin experiments were maintained at pH 4.0 to 4.2; bromelin and papain experiments, at pH 5.0. The temperature was 40° in all cases.

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STUDIES OF THE PHYSIOLOGICAL BEHAVIOR OF THE ACETYL DERIVATIVES OF THE OPTICAL ISOMERS OF HOMOCYSTINE; A BIOLOGICAL PROOF OF THEIR STEREOSTRUCTURE

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The resolution of homocystine with the isolation of both optical isomers has recently been reported by du Vigneaud and Patterson (1). When the two isomers were obtained, there was, of course, nothing to indicate which isomer corresponded in spatial configuration to the naturally occurring series of amino acids. This relationship was established by the conversion of each antipode to the corresponding isomer of methionine. The isomer which gave rise to *l*-methionine and possessed a specific rotation of $+76^\circ$ in HCl solution was designated as *l*-homocystine.

The present paper brings to bear a biological confirmation of this chemical proof of configuration. We also hope to show that this biological approach may afford the basis of a method of establishing which of the individual isomers of an amino acid that has not been isolated from natural sources corresponds in spatial configuration to the naturally occurring series of amino acids. Such a method would be of particular value where chemical proof of configuration might be extremely difficult. The biological method of approach to which we refer is based on the differential behavior in the animal organism of the optical isomers of the acetyl derivative of the amino acid.

Various studies have brought out the difference in availability for growth purposes of the optical isomers of the acetyl and formyl derivatives of various amino acids in which both of the enantiomorphic forms of the free amino acid are utilizable. It was

shown in the work of du Vigneaud, Sealock, and Van Etten (2) that, although animals on a tryptophane-free diet could utilize both *d*- and *l*-tryptophane for growth purposes, they could use only the levo isomer of the acetyl derivatives. The acetyl-*d*-tryptophane failed to promote growth. This observation was confirmed by Berg (3). Shortly thereafter Jackson and Block (4) demonstrated that the same relationship obtained in the case of methionine; both *d*- and *l*-methionine supported the growth of animals on a cystine-deficient diet, but of the formyl derivatives only the formyl-*l*-methionine was efficacious.

Since *d*-cystine had been found to be incapable of serving in lieu of *l*-cystine for growth purposes (5), no comparison could be made between the acetyl derivatives on this basis. It was found, however, that diacetyl-*d*-cystine was oxidized *in vivo* far less readily than *d*-cystine, whereas in the case of *l*-cystine no difference in the degree of oxidation of the free *l*-cystine and its acetyl derivative was observed (6). The amount of extra sulfate excreted in the urine after the administration of diacetyl-*d*-cystine was almost negligible. This same behavior was found in the *in vivo* oxidation of the formyl derivatives (6).

It would therefore appear that the acetyl or formyl derivative of an optical isomer of an amino acid belonging to the series foreign to the body is not available for supporting growth even when the free isomer itself is utilizable; and also that the acetyl or formyl derivative of the unnatural isomer is less readily oxidized than the corresponding free amino acid, whereas acetylation or formylation of the naturally occurring form does not influence to any appreciable degree its oxidizability or availability for growth. Hence either of these methods of approach should be useful in establishing which of two antipodes corresponds to the naturally occurring series of amino acids, and, by inference, in demonstrating the actual spatial configuration, since all the members of this series that have been studied have been shown to have the levo configuration regardless of the actual direction of rotation. In the case of homocystine both methods appeared to be applicable, although it is clear that in most instances only the oxidation approach would find application. In the case of sulfur amino acids oxidation of the sulfur could be utilized, whereas with non-sulfur-containing amino acids studies involving isolation

of the non-utilizable form from the urine would have to be resorted to.

The only observation in the literature not in agreement with the above thesis with respect to the acetyl derivatives of amino acids is that of Knoop and Blanco (7) on phenylalanine. However it has recently been shown that confusion existed in this study with regard to the identity of the isomers. It would appear from their work that the acetyl-*l*-phenylalanine was less readily oxidized than the acetyl-*d*-phenylalanine. As shown by du Vigneaud and Meyer (8) and confirmed by du Vigneaud and Irish (9), the acetyl derivative regarded by Knoop as that of the naturally occurring form was in fact the acetyl derivative of *d*-phenylalanine, the isomer foreign to the body. Their work, therefore, is in harmony with the work quoted above regarding the oxidizability of the enantiomorphic acetyl derivatives of amino acids.

As reported by Dyer and du Vigneaud (10), it was found that both of the optical isomers of homocystine were capable of serving in lieu of cystine for animals on a cystine-deficient diet. On the basis of the considerations just mentioned with regard to the effect of configuration on the utilization of acetyl derivatives of amino acids, we felt we should be able to ascertain biologically which of the isomers belonged to the naturally occurring series of amino acids. This isomer should be the one whose availability for growth and oxidizability *in vivo* would not be affected by acetylation. Experiments designed to test the validity of these postulations were therefore undertaken.

EXPERIMENTAL

The *d*- and *l*-homocystines were prepared from the corresponding *S*-benzyl derivatives obtained by the resolution of *dl*-*S*-benzylhomocysteine, as previously described by du Vigneaud and Patterson (1). The preparation of acetyl-*l*-homocystine was accomplished by the same procedure as that used by Hollander and du Vigneaud (11) for the corresponding cystine compound. The acetyl-*l*-homocystine, like acetyl-*l*-cystine, was not isolated in crystalline form. It was obtained as a white fluffy solid, appearing crystalline macroscopically but definitely non-crystalline

50 Optical Isomers of Acetylhomocystine

under the microscope. The material, dried at 60° *in vacuo*, had the following composition.

2.757 mg. substance: 0.199 cc. N at 34° and 757 mm.
 $C_{12}H_{10}O_6N_2S_2$ Calculated, N 7.99; found, 7.99

The anhydrous material possessed a specific rotation of $[\alpha]_D^{30} = -21.3^\circ$ and the neutral equivalent of the material checked with the theoretical value. The acetyl-*d*-homocystine prepared in like manner possessed a rotation of $[\alpha]_D^{31} = +21.5^\circ$ for a 1 per cent aqueous solution.

Growth Experiments—The basal diet employed in the growth studies was identical with that used in the study of the utilization of homocystine (12), having the following percentage composition: casein 5.0, dextrin 38.0, sucrose 15.0, salt mixture (Osborne and Mendel (13)) 4.0, agar 2.0, lard 19.0, milk vitamin concentrate (Supplee *et al.* (14)) 12.0, and cod liver oil 5.0. Control animals were maintained in each litter both on the basal diet alone, and on a supplement of *dl*-homocystine which was known to support growth. All the animals were kept on the basal diet for a fore period of from 4 to 5 days. During this period they lost from 9 to 18 gm. in body weight. This period is not included in Chart I.

In the first litter, at the end of the fore period, Rats 349 and 351 were continued on the basal diet alone throughout the experimental period. Rats 348 and 355 were given the basal diet supplemented with 0.335 per cent of homocystine for the following period of 36 days. Rats 345 and 352 were fed the basal diet supplemented with 0.44 per cent acetyl-*d*-homocystine and Rats 346 and 350 were maintained on the basal diet supplemented with the same percentage of acetyl-*l*-homocystine. This concentration is the equivalent in sulfur content of 0.3 per cent *l*-cystine or 0.335 per cent homocystine. At the end of 36 days all the animals were kept on the basal diet alone for a period of 8 to 12 days. The growth curves for this litter are presented in Chart I and the daily food consumption, including the fore period of the animals on the basal diet, is recorded in Table I.

The second litter of rats, on the same percentage basal diet, was used at a later date. One animal was kept on the basal diet alone, one was given a supplement of *l*-cystine, two rats were given acetyl-*l*-homocystine, and two were given acetyl-*d*-homo-

cystine. An attempt was made to administer the acetylhomocystine and cystine supplements in the form of dextrin pills. The

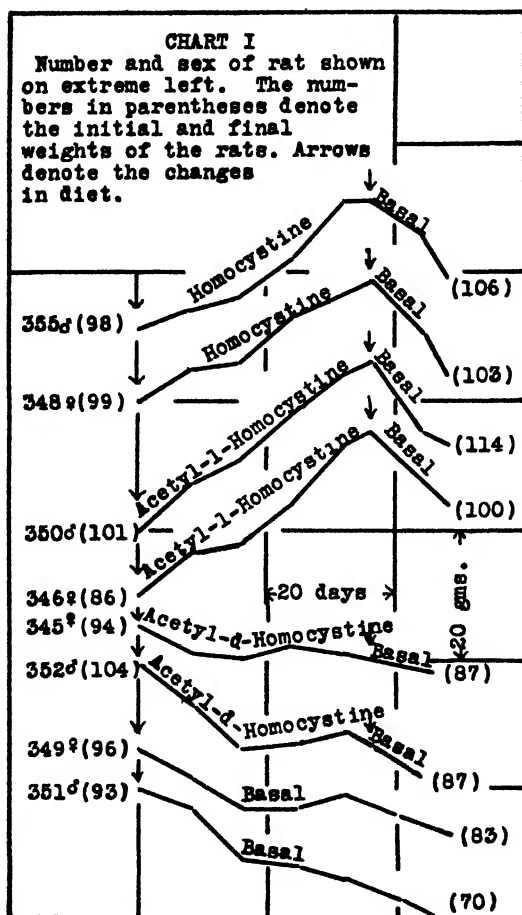


CHART I. Growth curves of the first litter, showing the comparative rates of growth of rats on a cystine-deficient diet supplemented with homocystine, with acetyl-l-homocystine, or with acetyl-d-homocystine.

animals refused to take the acetyl-d-homocystine pills on the 2nd day, so the supplements were again incorporated in the diet as was done with the first litter. The acetyl derivatives were

52 Optical Isomers of Acetylhomocystine

added to the basal diet in concentrations of 0.44 per cent, while *l*-cystine was added in a concentration of 0.3 per cent. Since the results completely duplicated those of the first litter, the growth curves for these animals have not been presented in order to conserve space.

TABLE I
Food Consumption

Rat No. and sex	Days	Daily supplement to basal diet	Average daily food consumption
			<i>gm.</i>
349 ♀	1- 4	Acetyl- <i>l</i> -homocystine	8.8
	4-40		7.2
	40-52		6.0
351 ♂	1- 4		8.3
	4-40		5.6
	40-52		3.8
346 ♀	1- 4		7.0
	4-40		8.2
	40-52		7.1
350 ♂	1- 4	Acetyl- <i>l</i> -homocystine	7.8
	4-40		9.1
	40-52		7.1
345 ♀	1- 4	Acetyl- <i>d</i> -homocystine	7.8
	4-40		7.9
	40-52		5.7
352 ♂	1- 4	Acetyl- <i>d</i> -homocystine	9.0
	4-40		6.8
	40-52		4.9
348 ♀	1- 4	Homocystine	8.5
	4-40		8.2
	40-52		5.6
355 ♂	1- 4	Homocystine	7.3
	4-40		8.5
	40-52		6.3

Oxidation Studies—The investigation of the oxidizability by the animal body of these acetyl derivatives was made according to the technique followed in the comparison of the oxidation of the dextro and the levo forms of diacetylcystine in the rabbit (6). The animals were kept in metabolism cages with a double false bottom to avoid admixture of feces with urine. A definite daily intake of Pratt's Rabbit Pellets was given. Analyses were carried

TABLE II

Analytical Results of Oxidation Studies for Individual Experiments

Day	Homocystine fed, 0.5 gm.	Total S	Total SO ₄ -S	Organic S	Total N	Day	Homocystine fed, 0.5 gm.	Total S	Total SO ₄ -S	Organic S	Total N
Rabbit 8, 2.3 kilos; 110 gm. Rabbit Pellets* daily						Rabbit 9—continued					
		mg.	mg.	mg.	gm.			mg.	mg.	mg.	gm.
1	Acetyl- <i>l</i> -	164	141	23	1.89	11	<i>d</i> -	135	116	19	1.57
2		166	150	16	1.84	12		144	121	23	1.71
3		167	147	20	1.87	13		140	118	22	1.65
4		238	196	42	1.82	14		218	171	47	1.76
5		161	140	21	1.84	15		149	127	22	1.41
6		159	138	21	1.84	20		122	102	20	1.59
7		163	140	23	1.79	21		122	105	17	1.57
8		163	142	21	1.87	22		122	105	17	1.61
9		242	207	35	1.73	23		209	125	84	1.76
10		159	138	21	1.69	24		139	107	32	1.56
11	<i>l</i> -	151	130	21	1.68	34	Acetyl- <i>d</i> -	124	106	18	1.56
12		153	132	21	1.69	35		135	115	20	1.59
13		147	129	18	1.73	36		136	116	20	1.64
14		255	186	69	1.76	37		215	129	86	1.63
15		152	129	23	1.64	38		142	118	24	1.56
21		135	117	18	1.64	Rabbit 10, 2.1 kilos; 110 gm. Rabbit Pellets daily					
22		143	125	18	1.69	1	Acetyl- <i>d</i> -	141	118	23	1.37
23		133	116	17	1.62	2		131	106	25	1.50
24		227	187	40	1.52	3		141	116	25	1.58
25		146	127	19	1.62	4		219	129	90	1.54
Rabbit 9, 2.9 kilos; 110 gm. Rabbit Pellets daily						5		135	98	37	1.33
1	Acetyl- <i>l</i> -	162	142	20	1.81	6		126	100	26	1.40
2		164	137	27	1.87	7		143	115	28	1.42
3		157	137	20	1.81	8		140	113	27	1.42
4		231	191	40	1.83	9		221	164	57	1.43
5		162	135	27	1.77	10		148	118	30	1.44
6		158	134	24	1.81	11		154	123	31	1.56
7		151	129	22	1.71	12		158	133	25	1.64
8		149	131	18	1.74	13		144	118	26	1.53
9		240	205	35	1.74	14		244	136	88	1.62
10		151	131	20	1.62	15		155	119	36	1.56

* Pratt's.

out on 24 hour specimens of urine which were obtained at a definite time each morning. At this time the bladder was emptied by gentle pressure upon the abdomen and the urine thus got added to that which had been collected from the cage. The compound dissolved or suspended in water was administered by means of a stomach tube before the daily ration was placed in the cage. Total sulfur was determined by Denis' (15) modifi-

TABLE III
Percentage Oxidation of Homocystine and Acetylhomocystine Isomers

Rabbit No.	Compound administered	Total S	Average total S of fore period	Increase in total S	SO ₂ -S found	Average SO ₂ -S of fore period	Increase in SO ₂ -S	Increased S ap- pearing as SO ₄	Administered S oxidized	S recovered
		mg.	mg.	mg.	mg.	mg.	mg.	per cent	per cent	per cent
8	<i>l</i> -Homocystine	242	162	80	207	140	67	84	56	67
9	"	240	153	87	205	131	74	85	62	73
8	"	227	137	90	187	119	68	76	57	76
9	Acetyl- <i>l</i> -homocystine	231	161	70	191	139	52	74	57	77
8	"	238	166	72	196	146	50	69	55	79
9	<i>d</i> -Homocystine	218	140	78	171	118	53	68	45	66
8	"	255	150	105	186	130	56	53	47	88
10	"	221	136	85	164	109	55	65	46	71
9	Acetyl- <i>d</i> -homocystine	209	122	87	125	104	21	24	23	96
10	"	219	138	81	129	113	16	20	18	90
9	"	215	132	83	129	112	17	20	19	91
10	"	224	152	72	136	125	11	15	12	80
Free <i>l</i> -cystine previously reported (6).....								82	67	82
Acetyl- <i>l</i> -cystine previously reported (6).....								80	63	79
<i>d</i> -Cystine previously reported (6).....								45	32	71
Acetyl- <i>d</i> -cystine previously reported (6).....								13	7	58

cation of Benedict's procedure (16). Total sulfates were estimated by the Folin (17) method. Total nitrogen determinations were carried out by the macro-Kjeldahl procedure.

The individual experiments with the isomers are given in Table II. The data from the individual experiments are summarized in Table III together with the calculations of the percentage oxidation of the compounds. For the data in Table III, the in-

crease in total sulfur was obtained from the difference between the average value of the total sulfur during the fore period and the value found during the 24 hour period after feeding the compound. The increase in total sulfates was obtained in a similar way. The percentage of oxidation is expressed as the percentage of the extra sulfur excreted which appeared in completely oxidized form, and as the percentage of the total sulfur administered which appeared completely oxidized.

DISCUSSION

As clearly shown in Chart I, the isomer which we found upon reduction and subsequent methylation to yield *l*-methionine, and which we therefore called *l*-homocystine, was still capable, after it had been acetylated, of supporting the growth of animals on a cystine-deficient diet. On the other hand the acetyl derivative of the other optical isomer of homocystine, diacetyl-*d*-homocystine, was found to be incapable of serving in this capacity, although *d*-homocystine itself is capable of supporting growth (10). In the light of the discussion presented in the introduction of this communication, the results of these experiments offer convincing confirmation of our earlier conclusions with regard to the spatial configuration of the optical isomers of homocystine, which were based on chemical evidence.

The oxidation experiments yielded results in complete agreement with those of the growth studies. As shown in Tables II and III, a vast difference in the oxidizability of the enantiomorphic acetyl derivatives was disclosed, whereas little difference was found to exist between the free isomers. Acetylation of the isomer which we had considered as belonging in configuration to the natural series of amino acids did not impair its oxidation. A comparison of the data in Table III shows that the percentage of the extra sulfur which appeared in the completely oxidized form after the administration of the diacetyl-*l*-homocystine did not differ greatly from that which was found following the administration of the free amino acid, whereas much less extra oxidized sulfur appeared in the urine after the feeding of the acetyl derivative of the *d*-homocystine than after the *d*-homocystine itself. The total recovery of sulfur in the urine was approximately the same after the feeding of *d*-homocystine and

its acetyl derivative, an observation which rules out the question of absorption. The urines were also tested for disulfide and a considerable amount was found after the administration of the acetyl-*d*-homocystine, which again points to the difficulty in oxidation.

There is some suggestion in the data that *l*-homocystine is somewhat more readily oxidized than the dextro isomer. The difference, however, was not nearly as marked as that which had been found to exist between *d*- and *l*-cystine (18).

The outcome of these studies gives us increased confidence that this biological method of approach will prove of valuable aid in establishing which of the optical isomers of an amino acid not existing in nature belongs in spatial configuration to the naturally occurring series of amino acids. It will be of interest to extend the oxidation study to pentocystine and hexocystine to see if such a difference in oxidizability holds true in the case of the optical isomers of their acetyl derivatives. This biological approach may offer some basis for deciding which of the two optically active isomers of these homologues of cystine belongs to the naturally occurring series of amino acids in stereostructure. In these instances the chemical approach to establishing configuration would be extremely difficult.

SUMMARY

The spatial configuration of the optical isomers of homocystine which was assigned to them on the basis of chemical evidence has been confirmed biologically.

It has been shown that acetylation of *l*-homocystine neither affects appreciably its availability for growth purposes nor to any great degree its oxidation *in vivo*, whereas acetylation of *d*-homocystine prevents its utilization for growth purposes and hinders greatly its oxidation. The significance of these results to the stereostructure of the isomers is discussed in the light of previous work on the acetyl derivatives of amino acids.

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THE PREPARATION AND PURIFICATION OF EXTRACTS CONTAINING THE GONAD-STIMULATING HORMONE OF PREGNANT MARE SERUM

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Cole and Hart (1) have observed that during a limited period of pregnancy there is present in the blood serum of mares a high concentration of a substance which stimulates the gonads. In this and subsequent reports, Cole and Hart and their coworkers have given an excellent description of the physiological properties of this hormone. For a review of this literature, together with numerous reports from other laboratories, we would refer to Goss and Cole (2), Catchpole and Lyons (3), and Hamburger (4), in which papers complete bibliographies are given.

By comparison with the voluminous physiological literature concerning the mare serum hormone, relatively few articles have been published with regard to chemical purification of the active substance. Goss and Cole (2) have fractionally precipitated mare serum with sodium sulfate, the active material being precipitated with the pseudoglobulin fraction. This procedure accomplished approximately a 10-fold purification of the hormone but introduced the difficulty of removing by dialysis the salts associated with the active precipitate.

Evans, Gustus, and Simpson (5) have accomplished a high purification of the mare serum hormone by adsorption on aluminum hydroxide. The final active product was obtained as an aqueous solution which was freed of salts by dialysis. Aluminum hydroxide adsorption was further studied in our laboratories by Gustus, Meyer, and Woods (6), and adapted to the fresh plasma.

Catchpole and Lyons (3) have applied acetone precipitation to mare serum but apparently their procedure failed to accomplish

any appreciable separation of the hormone from the serum proteins.

Meyer (7) has described a method of purifying the gonad-stimulating principle of mare serum by precipitation of dilute butanol extracts with acetone. After removal of proteins by precipitation with aluminum sulfate the final active product contained large amounts of inorganic salts which offered great difficulty in attempts at further purification.

The present studies were undertaken with the purpose of developing a practical method for obtaining the gonad-stimulating hormone free from the serum proteins in the form of a dry, stable, soluble powder which could be used for preparing sterile solutions for further laboratory and clinical investigation.

EXPERIMENTAL

Biological Assay Method—Follicle stimulation, ovulation, corpus luteum formation, and estrus changes in the uterus and vagina may be produced in immature female rats by the injection of the mare serum hormone. For the purpose of quantitative assay we have employed a method based upon the increased ovarian weight of immature rats injected with the hormone. Female rats of our Wistar strain colony, weighing 30 to 40 gm. at 21 to 23 days of age, are injected subcutaneously on each of three successive days with 1 cc. doses of suitable dilutions of the hormone in physiological saline. 96 hours after the first injection the rats are sacrificed and the ovaries dissected free of oviducts and bursæ, examined for corpora lutea, and weighed. Our rat unit is defined as the total dose of hormone which, administered as described above, will produce at autopsy a pair of ovaries weighing 65 mg., which is approximately 5 times the weight of the ovaries of the uninjected controls. The ovarian weight of 65 mg. has been chosen as the unit response because it represents a point of high sensitivity in a region where the response is almost a straight line function of dosage, thus yielding a higher accuracy than can be obtained by working at a much lower dosage level.

A satisfactory procedure of assay is to estimate the approximate strength of a given extract by a series of preliminary injections and, on the basis of the results obtained, inject two groups of ten to twenty rats each with amounts estimated respectively to be

greater and less than the 1 unit dose by approximately 25 per cent. The dose necessary to produce an ovarian weight of 65 mg. is determined by interpolation and, if desired, this may be checked by a further test. More than 3000 rats have been used in following the hormone activity in the fractionations described below. In our experience with repeated assays on stable fractions, this method has yielded reproducible results within an experimental error of ± 10 per cent.

We have made a number of comparative assays in order to evaluate approximately our unit in terms of other methods of assay which have appeared in the literature. The results indicate that our unit is approximately 10 times as large as the rat unit of Cole and Saunders (8) which has been described as one-twelfth of the total dose necessary to produce ovaries weighing 55 to 69 mg. under their experimental conditions. Also, our results indicate that our unit is approximately 2 to 3 times as large as the rat unit employed by Meyer (7), and 10 times as large as the mouse unit of Evans, Gustus, and Simpson (5), both of which are based upon a doubling of ovarian weight.

Starting Material—Blood was collected from pregnant mares into sterile bottles containing sufficient 5 per cent sodium citrate to prevent coagulation and the separated plasma kept frozen until used. In agreement with the findings of Cole and Hart (1) we have found, in collecting blood from forty-nine mares, that the most active plasmas are obtained in the neighborhood of the 65th day of pregnancy.

Method of Fractionation—In precipitation methods previously described in the literature the hormone has been precipitated from the serum together with a large part and in some cases all of the serum proteins, thus yielding a bulky protein precipitate from which the hormone had to be separated by various subsequent procedures. In the method here described we have worked on the opposite principle of starting with the fresh plasma and removing the inactive proteins as completely as possible by methods which leave the hormone almost quantitatively in the soluble phase and finally precipitating the hormone in the form of a highly active powder which is substantially free from serum proteins.

In a series of preliminary experiments the plasma at a pH of 7 to 7.5 was precipitated with alcohol and acetone at concentra-

tions of 30, 40, 50, 60, 70, 80, and 90 per cent and the amount of hormone determined in the precipitate and supernatant liquid in each case. It was found that the hormone remained in solution in alcohol up to 60 per cent and acetone up to 50 per cent, but at higher concentrations of acetone or alcohol, such as 70 to 80 per cent, the hormone appeared almost quantitatively in the protein precipitate. Thus, by precipitating the plasma with 60 per cent alcohol or 50 per cent acetone, the greater part of the plasma proteins is removed in the bulky, inactive precipitate, leaving most of the hormone in the clarified supernatant solution.

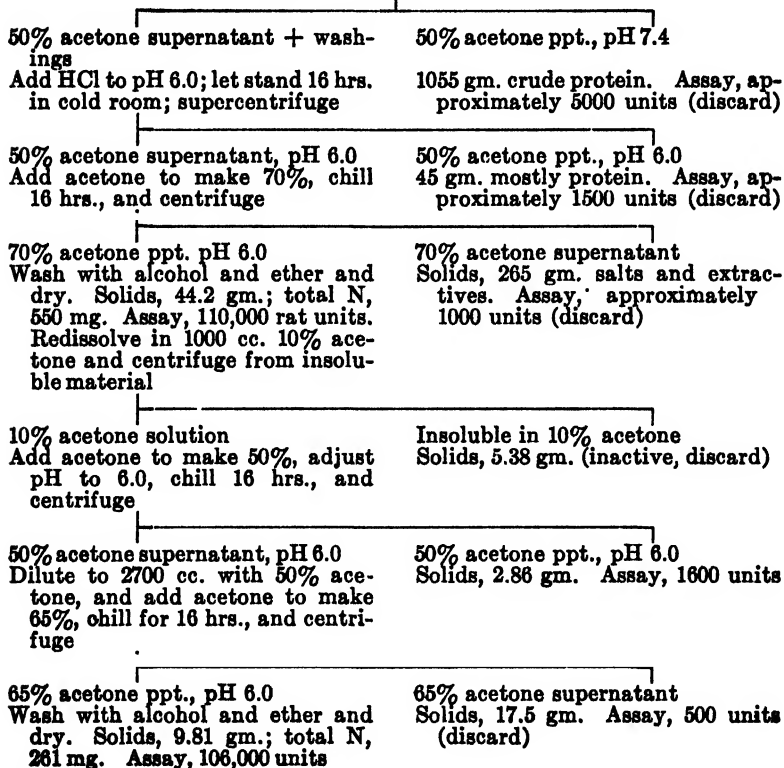
Before precipitating the active hormone fraction by increasing the alcohol or acetone concentration, we have found it advantageous to adjust the pH to approximately 6, at which a flocculent precipitate of inactive protein material separates, leaving the hormone almost quantitatively in the supernatant solution. This step greatly improves the purity and solubility of the active hormone fraction, which is next precipitated by increasing the alcohol or acetone to approximately 70 per cent. The active precipitate is washed with alcohol and ether and dried *in vacuo*, yielding a white, water-soluble powder containing from 70 to 90 per cent of the hormone present in the original crude plasma. The active fraction obtained in this way often contains considerable amounts of sodium citrate, causing appreciable variability in activity in terms of rat units per mg. of solids (see Table I). This salt and also additional nitrogen-containing substance can be removed without significant loss of hormone by redissolving the active precipitate in water and repeating the fractionation as previously described, but making the final precipitation at 65 per cent acetone.

The process for a typical fractionation of 16.5 liters of plasma is outlined in the accompanying flow sheet, giving quantitative distribution of solids and hormone at various stages of purification. The final product obtained by this procedure is easily soluble in water, making possible solutions containing more than 400 rat units per cc. When compared with the original plasma, this active fraction represents approximately a 130-fold purification on the basis of total solids and a 560-fold purification on the basis of total nitrogen. This material is substantially free from serum proteins, as is shown by both chemical and immunological studies

and, since it can be collected as a sterile powder by using aseptic precautions at the time of final drying and transfer, it forms a satisfactory basis for preparing sterile aqueous solutions of the hormone. Fractions of this grade of purity have proved to be satisfactory for clinical studies.

*Flow Sheet of Fractionation Procedure for Preparation of
Gonadotropic Hormone*

16.5 liters citrated blood plasma (assay 119,000 rat units); adjust pH to 7.4 and add 16.5 liters of acetone; let stand 16 hrs., separate precipitate and wash with 16.5 liters of 50% acetone



In Table I we have summarized data on the assay, yields, and stability of a number of preparations of different grades of purity obtained by the process outlined in the accompanying flow sheet. Fractions which were obtained by a single precipitation represent

70 per cent acetone precipitates at pH 6.0, as outlined midway in the fractionation procedure; those labeled as twice precipitated (Nos. 122-C-18 and 70-C-18) represent 65 per cent acetone precipitates at pH 6.0 obtained as outlined at the end of the flow sheet. Preparation 26-C-18 was obtained by dissolving in water and precipitating the active fraction a third time. Reassays after periods of 3 to 11 months show that these dried preparations possess a satisfactory stability when stored in corked vials at room temperature, thus providing ideal material for chemical studies and further purification.

Some Properties of the Dried Hormone Preparation—For these studies Preparation 144-C-16 was used, which is described in

TABLE I
Assay, Yields, Purity, and Stability of Dried Hormone Fractions

Preparation No.	No. of times pptd.	Hormone recovered from plasma	Assay per mg. solids	Total N per unit	Age at reassay	Reassay per mg. solids
		per cent	rat units	mg.	mo.	rat units
130-C-16	1	87	1.78	0.007	11	1.59
93-C-16	1	88	2.08	0.024	11	2.08
144-C-16	1	75	3.00	0.008	9	2.82
104-C-16	1	73	5.55	0.016	11	5.82
70-C-18	2	89	10.70	0.0025	3	8.70
122-C-18	2	65	22.20	0.0025		
26-C-18	3	82	19.60	0.004	6	19.20

Table I. This product is comparatively low in nitrogen but contains a considerable amount of sodium citrate which did not interfere with the determinations.

Heat Stability—A solution assaying 1 unit per cc. was divided into aliquots and heated for 30 minutes at temperatures of 60°, 70°, 80°, and 100°. The amount of hormone destroyed at pH 6.0, 7.0, and 8.0 respectively was as follows: at 100°, complete destruction in all cases; at 80°, 96, 88, and 73 per cent; at 70°, 55, 8, and 11 per cent; at 60°, no destruction. These results indicate a greater heat stability in neutral or slightly alkaline solution.

Formaldehyde—300 units of hormone were exposed to 4 per cent formaldehyde for 3 hours at pH 8 according to a procedure similar to that of Maxwell and Bischoff (9) who worked with an anterior

pituitary fraction. Approximately 84 per cent of the hormone was destroyed by this procedure. Injection of the portion of the hormone which escaped inactivation by formaldehyde over a range of dosage between 0.25 and 2.5 unit in parallel with equivalent unit doses of the untreated control yielded similar results with respect to ovarian weight, follicle stimulation, and corpus luteum formation, thus giving no evidence of a selective destruction of a luteinizing fraction by formaldehyde.

Treatment with Enzymes—The resistance of the purified hormone to the action of pepsin, trypsin, emulsin, and invertin was studied, each enzyme experiment being paralleled by a control in which a heat-destroyed enzyme solution was substituted. When incubated at 40° with trypsin at pH 7.5 and 8.7 the hormone was 70 to 75 per cent destroyed after 1 hour and completely destroyed after 6 hours, the controls showing no loss during this procedure. These results are in agreement with those of Cole and coworkers (2, 10) and of Evans, Gustus, and Simpson (5).

Exposure to an active pepsin for 1 hour at 40° at pH 3.0, 2.0, and 1.17 resulted in losses of 35, 75, and 100 per cent respectively, but this destruction is due to acidity rather than to a specific effect of pepsin, since the heated enzyme controls at the same acidities showed a similar loss of potency. In aqueous solution, the hormone is quickly destroyed by strong acids. Incubation at 40° for 1 hour with invertin or emulsin at pH 6.5 did not have any destructive influence on the hormone.

Further Purification of Active Hormone Fraction—For this purpose, 89,000 units of Preparation 122-C-18, which is described in Table I, was used as starting material since it already represents a product of comparatively high purity. This was dissolved in 650 cc. of 10 per cent acetone and subjected to the procedure previously outlined, yielding 80,000 units of hormone in the form of a 65 per cent acetone precipitate weighing 1.288 gm. This was redissolved in 155 cc. of 10 per cent alcohol and fractionally precipitated with alcohol at pH 5, yielding 749 mg. of a white, water-soluble powder. This product was assayed by the method described above, total nitrogen was determined by micro-Kjeldahl procedure, ash was determined, and specific rotation was taken on an 0.2 per cent solution in 10 per cent alcohol at pH 6.75, with the following results: assay, 57,600 units = 77 units per mg. of

solids; total N, 9.62 per cent = 0.00125 mg. per unit; $[\alpha]_D^{25}$ (in 10 per cent ethyl alcohol) = -39° ; ash, 5.0 per cent of solids.

In the course of this purification a small fraction was obtained by precipitating with alcohol at pH 4.5, which weighed 7 mg. and assayed 140 units per mg. Reprecipitation of this fraction from 70 per cent acetone failed to increase the activity. In reworking a fraction from an entirely different lot of plasma by the process previously described, another small precipitate was obtained weighing 10.3 mg. and assaying 140 units per mg. This product contained 10.8 per cent total nitrogen corresponding to 0.00074 mg. per unit. Since the original plasma contains not less than 12 mg. of solids and 1.4 mg. of total nitrogen per unit of hormone, these most purified fractions, containing 0.0071 mg. of solids and 0.00074 mg. of total nitrogen per unit, represent approximately an 1800-fold purification of the hormone as it exists in the raw plasma. As little as 0.1 to 0.2 rat unit of the purified hormone, when injected into 21 to 23 day-old rats as described above, will sufficiently stimulate the ovaries so as to produce canalization of the vagina. In a previous report from this laboratory (11), using the method of Curtis and Doisy (12), we have shown that it requires 0.001 mg. of crystalline theelin or 0.00025 mg. of theelol to produce a similar vaginal reaction. Although this end-reaction is produced indirectly by the gonadotropic hormone and directly by the estrogenic substances, this comparison shows that our most highly purified fractions closely approach the range of physiological activity of the pure estrogenic hormones with respect to the immature rat.

We are now engaged in attempts at preparing substantial amounts of this hormone in a state of maximum purity with the purpose of studying its chemical composition.

SUMMARY

A method is described for preparing highly purified extracts of pregnant mare plasma containing the gonadotropic hormone. This procedure consists essentially of fractional precipitation with acetone or alcohol, removal of impurities by proper adjustment of acidity, and final precipitation of the hormone fraction in the presence of increased concentration of acetone or alcohol. Yields of hormone as high as 60 to 90 per cent have been regularly

obtained from the crude plasma in the form of a product representing a 130-fold purification. The hormone fractions are obtained as dry, white, water-soluble powders which are remarkably stable, furnishing a satisfactory basis for preparing sterile solutions for laboratory and clinical studies. By precipitation at the isoelectric point in the presence of alcohol or acetone, small amounts of two active fractions were obtained assaying 140 rat units per mg. when tested by a biological assay method which is described; this activity represents approximately an 1800-fold purification in terms of the original plasma.

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ISOLATION OF VINYL ETHER (DIVINYL OXIDE) FROM HUMAN TISSUES

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A quantitative method is presented for the recovery of vinyl ether (divinyl oxide) from human tissues. The substance dealt with was not pure vinyl ether but the Merck preparation which is sold for anesthetic purposes under the trade name of vinethene. The latter consists of vinyl ether, 3.5 per cent absolute alcohol, and 0.01 per cent of a non-volatile oxidation inhibitor.

Vinethene is a clear, practically colorless liquid, with a slight purplish fluorescence. The pungent odor is characteristic and increases in strength with the age of the sample. It is highly inflammable, explosive, and very unstable in the presence of light and heat. It is less soluble in water than is ethyl ether. The boiling point is 28–31° (Merck).

Rectification of Vinyl Ether from Aqueous Solution

The apparatus used was essentially that of Gettler and Siegel.¹ Solutions were prepared by dissolving 1.0 cc. and 0.5 cc. of vinethene in 1000 cc. of aqueous solution (0°), and samples withdrawn for rectification. Vinethene and glassware used in handling the solution were packed in cracked ice before use. Approximately 1.0 gm. of granulated zinc was added to the sample taken for analysis, the receiving vessel placed in a bath of solid carbon dioxide in acetone, and distillation carried out as follows: (a) The flask was heated with a microburner through wire gauze with an asbestos center, the flame so regulated that the contents reached a gentle boil after 70 to 75 minutes of heating; (b) when

¹ Gettler, A. O., and Siegel, H., *Arch. Path.*, **19**, 208 (1935).

steam began to rise in the rectification tube, the flame was controlled to permit approximately 15 minutes to elapse before the top of the tube became hot; (c) heating was continued until steam had passed the bend in the delivery tube. The receiving tube was removed, stoppered, and placed in an ice and water bath. When ice in the former had melted, the volume of vinyl ether (upper layer) was observed.

It is necessary that the rectification tube be thoroughly dried (alcohol followed by heat) before use, to prevent ice from clogging

TABLE I
Recovery of Vinyl Ether from Aqueous Solution

Determination No.	Vinethene added	Vinyl ether recovered	Vinyl ether lost	Vinyl ether recovered
	cc.	cc.	cc.	per cent
1	0.25	0.15	0.09	62.5
2	0.25	0.15	0.09	62.5
3	0.25	0.22	0.02	91.7
4	0.25	0.22	0.02	91.7
5	0.25	0.25	0.01	95.8
6	0.25	0.23	0.01	95.8
7	0.25	0.23	0.01	95.8
8	0.25	0.22	0.02	91.7
9	0.25	0.23	0.01	95.8
10	0.25	0.23	0.01	95.8
11	0.125	0.11	0.01	90.9
12	0.125	0.12	0.001	99.1
13	0.125	0.11	0.01	90.9
14	0.125	0.12	0.001	99.1
15	0.125	0.12	0.001	99.1

the narrow portion of the delivery tube. Very soon after heating is begun, long before the solution has reached a boil, the more volatile vinyl ether appears as fine droplets, condensation taking place in the cold rectifying tube. If the droplets remain stationary until boiling is reached, they represent simply water vapor which was not driven out in the drying operation.

The yields and percentage recovery in Table I represent vinyl ether, not vinethene, and, as such, appear corrected for the 3.5 per cent absolute alcohol contained in vinethene. In Determinations 1 and 2 (Table I), the heating time was shortened 15 to 20

minutes, in accordance with the time found sufficient here for the maximum yield of ethyl ether and benzene. Ethyl chloride like vinyl ether was found to require the longer heating time for maximum yield.

Recovery of Vinyl Ether from Human Tissues

Aqueous solution of vinethene, prepared as described above was added to 500 gm. of finely ground human brain. (alcohol- and ether-

TABLE II
Recovery of Vinyl Ether from Human Tissues

Determination No.	Vinethene added	Vinyl ether recovered	Vinyl ether lost	Vinyl ether recovered
	cc.	cc.	cc.	per cent
1	0.40	0.22	0.17	56.4
2	0.40	0.23	0.16	58.9
3	0.40	0.23	0.16	58.9
4*	0.40	0.22	0.17	56.4
5	0.40	0.22	0.17	56.4
6	0.40	0.23	0.16	58.9
7	0.25	0.12	0.12	48.0
8	0.25	0.14	0.10	58.3
9	0.25	0.14	0.10	58.3
10*	0.25	0.14	0.10	58.3
11	0.25	0.13	0.11	54.2
12	0.25	0.13	0.11	54.2
13	0.25	0.15	0.09	60.0
14†	0.165	0.10	0.06	62.5
15	0.165	0.10	0.06	62.5
16	0.165	0.11	0.05	68.7
17	0.165	0.10	0.06	62.5

* 500 gm. of liver (human) were used in place of brain.

† 500 gm. of lung (human) were used in place of brain.

free), mixed with 500 cc. of ice-cold water, 1.0 cc. of liquid petrolatum added, and the system steam-distilled, with the apparatus described by Gettler and Siegel.¹ 250 cc. of distillate were collected and subjected to rectification as described above. The results appear in Table II. Boiling points determined on ten of the analyses listed ranged between 25.6° as a minimum and 26.4° as a maximum. The average was 26.0°.

The reason for loss in vinyl ether was investigated, and the con-

clusion reached that some vinyl ether is destroyed during the process of steam distillation. When vinethene is added to the steam distillate, and rectified, the loss in vinyl ether is of the same order of magnitude as the loss encountered in the rectification of vinyl ether from aqueous solution. Ground brain tissue (500 gm.) in 500 cc. of water was steam-distilled, 250 cc. of distillate collected, small quantities of vinethene added to the latter, and the resultant solution rectified. The results obtained appear in Table III.

It was found impossible to recover vinyl ether from tissue by vacuum distillation. The vapor pressure of the substance is presumably too high to apply such a procedure to the quantities dealt with. A further attempt was made to avoid steam distilla-

TABLE III
Rectification of Vinyl Ether from Brain Liquor

0.20 cc. of vinethene was added in each case.

Determination No.	Vinyl ether obtained	Vinyl ether lost	Vinyl ether recovered
	<i>cc.</i>	<i>cc.</i>	<i>per cent</i>
1	0.19	0.003	98.4
2	0.18	0.013	93.2
3	0.18	0.013	93.2
4	0.19	0.003	98.4
5	0.19	0.003	98.4

tion by placing the mixture of ground tissue, vinethene solution, granulated zinc, and liquid petrolatum in the rectification flask, and proceeding as for the aqueous solution. Tissue, however, clogged the rectification tube, and sufficient back-pressure developed to ruin the experiment.

The author wishes to thank Dr. A. O. Gettler of Bellevue Hospital for the material and facilities extended in connection with this work.

SUMMARY

1. A micromethod for the isolation of vinyl ether from human tissues is described. The average recovery from 500 gm. of tissue containing 0.16 to 0.39 cc. of vinyl ether is 58.2 per cent.

2. Recovery of vinyl ether from aqueous solution is described. The average recovery from 0.12 and 0.24 cc. is 90.5 per cent.

ON GLYCOPROTEINS

III. THE POLYSACCHARIDES FROM PIG GASTRIC MUCOSA

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Only recently has attention been attracted to the biological properties of mucoproteins and of the polysaccharides derived from them, because heretofore they have been considered as serving a purely tectonic or protective function. However, an interesting relationship has been pointed out between the polysaccharides of vitreous humor, umbilical cord, and streptococcus (1). Another biological relation exists between the blood Group A factor and the polysaccharide from gastric mucin.

From the stomach wall of the pig, Levene and López-Suárez (2) obtained, after alkaline hydrolysis, a substance which they called mucoitinsulfuric acid. The analytical data reported were not in good agreement with the theoretical values for such a compound; *e.g.*, S found, 1.5 per cent; calculated, 5.3 per cent. Komarov (3) likewise obtained from the gastric juice of dogs with gastric fistula a similar polysaccharide. However, only nitrogen, sulfur, and reducing values were recorded; the amino sugar was not isolated nor the substance otherwise characterized.

It will be shown in this paper that pig gastric mucin yields two different polysaccharides, one acid, the other neutral. The acid polysaccharide contains hexuronic acid, acetylglucosamine, and sulfuric acid in amounts agreeing reasonably well with the theoretical values for mucoitinsulfuric acid. The neutral polysaccharide is composed of acetylglucosamine and galactose. It is of biological interest, since it is one of the most active blood Group A preparations yet isolated. It forms highly viscous solutions, and the mucoproteins containing this sugar seem to be responsible for the slimy nature of gastric mucin.

EXPERIMENTAL

The work started with a commercial sample of gastric mucin¹ prepared by scraping off the mucosa of pig stomach, subjecting the tissue to peptic hydrolysis, and precipitating the material from the solution with alcohol. It contained 10.2 per cent of nitrogen and 12.2 per cent of hexosamine (corrected for ash, 4.1 per cent, and moisture, 2.4 per cent) and formed very viscous turbid solutions. The purification of this commercial sample was difficult owing to its ability to act as a protective colloid and to the presence of peptone.

In our first experiments the gastric mucin was hydrolyzed with alkali, as in the method of Levene and López-Suárez (2). After fractionation of the hydrolysate with glacial acetic acid, alcohol, and acetone-ether mixtures, acid fractions were obtained, having equivalent weights of between 400 and 500,² 12 to 25 per cent uronic acid calculated as hexuronic acid, and 10 to 20 per cent hexosamine. The fractions were obviously heterogeneous and partly decomposed. They gave an immediate red color with Ehrlich's reagent, similar to that described by Blix for the sugar from submaxillary mucin (4). The uronic acid concentration did not run parallel to the titratable acid groups. From these experiments we concluded that an acid sugar containing hexosamine existed in the material. Fractionation with organic solvents after alkaline hydrolysis increased the percentage of this sugar in the precipitate.³

It was obvious that drastic treatment with strong alkali caused much decomposition and unsatisfactory products. Subsequently it was found that milder conditions were more successful; these led us to the recognition of the two polysaccharides present.

Preparation of Neutral Polysaccharide—In the preparation of the

¹ We wish to thank Dr. David Klein of The Wilson Laboratories, Chicago, for the gift of this material.

² In these and all other preparations, all fractions were precipitated from acid solution in order to obtain free uronic acid groups. They were titrated both in the absence of and in the presence of formaldehyde.

³ The increase in acid groups in such material was formerly thought of as a possible saponification of esterified uronic acid groups (5). The acid sugar is more stable towards alkali and is more readily precipitated than the neutral polysaccharide, thus giving an apparent increase in uronic acid after precipitation with organic solvents.

neutral polysaccharide, the use of alkali could not be entirely avoided because simple precipitation by alcohol gave a highly viscous and difficultly soluble material. The original preparation of gastric mucin was treated with 2 per cent Na_2CO_3 for 15 minutes at 70° . This was sufficient to disaggregate the sugar and apparently was not harmful, as was later shown by the blood group reaction of the samples isolated from it.

Various procedures were followed to obtain the neutral polysaccharide. For removing the bulk of the nitrogenous impurities the following methods were usually used: (1) shaking of the neutral solutions with a mixture of chloroform and amyl alcohol, and precipitation by $\text{Zn}(\text{OH})_2$ (this was not as successful here as in earlier work (6), owing to the low molecular weight of the nitrogenous material and the protective action of the sugar); (2) fractionation with lead acetate in neutral and alkaline solution and fractionation from alcohol, glacial acetic acid, and acetone; (3) adsorption from acid solution with kaolin or Lloyd's reagent (this proved the most effective method). The precipitation of nitrogenous material by benzylation or carbobenzoxylation, or by alkaline copper salts was unsuccessful.

For the separation of the acid fraction from the neutral polysaccharide, the ability of acid polysaccharides to form protein salts in acid solution (6) was used. In the early stages this was effected by acidification of the crude solution and removal of the precipitate, which contained a large part of the acid sugar in combination with protein; later, a gelatin solution was added, the mixture acidified with acetic acid to 2 per cent, and the precipitate removed.

An example of a method of preparing the neutral polysaccharide follows. (1) 20 gm. of gastric mucin were dissolved in 500 cc. of H_2O with warming and neutralization. 10 gm. of Na_2CO_3 were added and the material was kept at 70° for about 15 minutes. (2) The solution was cooled, acidified with glacial acetic acid to a pH of about 5, and allowed to stand in the cold overnight. (3) The precipitate was removed by centrifugation. The supernatant solution was brought to 5 per cent H_2SO_4 , shaken with 100 gm. of Lloyd's reagent for a few minutes, and centrifuged. The sediment was extracted once with 300 cc. of H_2O , and the supernatant solutions were combined. (4) 2 volumes of alcohol were added,

and the precipitate was washed in alcohol⁴ and dissolved in 200 cc. of H₂O. 2 gm. of gelatin in 50 cc. of H₂O were added, followed by acetic acid to a concentration of 2 per cent. The mixture was placed in the ice box overnight. (5) After centrifugation, the residue was washed with a small amount of H₂O and the combined supernatant solutions were poured into 2 volumes of alcohol. (6) The precipitate was washed with alcohol and dissolved in 500 cc. of H₂O. About 50 cc. of neutral 25 per cent lead acetate were added, and the precipitate was removed. (7) N NH₄OH was added to the supernatant solution to a pH of about 9 (red to phenolphthalein), followed by 20 cc. of basic 25 per cent lead acetate. The caseous precipitate was washed with H₂O and suspended in 200 cc. of H₂O. The suspension was heated to 60° and CO₂ was passed through it. PbCO₃ was removed. If the solution remained turbid, N NH₄OH was again added and the treatment with CO₂ was repeated. (8) The solution was acidified with glacial acetic acid, and 3 volumes of alcohol were added. The precipitate was washed with alcohol, dissolved in 10 times its weight of H₂O,⁵ and the solution was precipitated by adding 12 times its volume of glacial acetic acid. (9) The precipitate was washed with alcohol, acetone, and ether and dried.

The above method yielded 3.00 gm. with the following analysis:⁶ N, 5.4 per cent; hexosamine, 36.2 per cent; uronic acid, 1.1 per cent; acetyl, 10.2 per cent; moisture, 3.8 per cent; ash, 4.2 per cent.

For further purification, 2.92 gm. of this powder were dissolved in 150 cc. of H₂O, 30 cc. of 8 per cent zinc acetate were added, and the solution was adjusted to maximal precipitation with N NaOH. The supernatant solution was poured into 3 volumes of alcohol. The precipitate was washed and dissolved with warming in 20 cc.

⁴ Since the precipitates were of rubbery consistency, and changed to a hard brittle mass in increasing alcohol concentrations, it was necessary every time to wash by triturating with alcohol in a porcelain mortar.

⁵ A test with mercuric acetate in acetic acid showed no more precipitation at this stage.

⁶ The analytical procedures followed are described in Paper II of this series (6). In the Kjeldahl determination, 2 drops of 30 per cent H₂O₂ were always added to aid the oxidation. Samples were hydrolyzed in 2 cc. of 4 N HCl for reducing sugar and amino sugar determinations. All analytical values given are corrected for ash and moisture (loss at 90°).

of H_2O . 200 cc. of glacial acetic acid were added with vigorous shaking. The precipitate (A) was washed and dried (yield, 0.245 gm.). To the supernatant solution above, 200 cc. of glacial acetic acid were added and the resulting precipitate washed, dried (yield, 2.177 gm.), and dissolved in 20 cc. of H_2O . 200 cc. of acetone were added in portions and the precipitate (B) was washed and dried (yield, 1.550 gm.). To the turbid supernatant acetone solution, 200 cc. more of acetone and a few cc. of sodium acetate in alcohol were added. The precipitate (C) was washed and dried (yield, 0.4896 gm.). The analyses⁶ of these substances

TABLE I
Analyses of Neutral Polysaccharide

Preparation No.	Nitrogen	Hexosamine	Acetyl	Hexuronic acid	Carboxyl	Moisture	Ash	$[\alpha]_D$
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	degrees
A	5.4	33.3	10.0	1.2	0	6.2	2.3	
B	5.4	34.0	10.8	0.5	0.5	5.0	1.2	
C	5.0	34.3	10.9	1.1	0.9	5.3	0	-4.9
1	4.8	30.8	10.7	6.1	2.4	1.3	1.2	-8.0
2	6.2	31.6	10.8	2.7	2.9	7.7	0.9	
3*	5.6	37.3	11.1	1.3	0.4	5.6	0	
4†	5.1	35.8	14.8	3.3	0.8	6.5	0.8	
5‡	5.2	38.6	10.9	2.0	0.4	7.3	0.9	+8.0
6	3.9	31.8	16.8	0.3		6.1	1.6	
7	3.3	34.0	9.2	1.0		2.5	2.0	+8.7
8	5.3	37.5	9.8	0.4	0	2.8	1.3	-2.2

* Prepared from commercial pepsin.

† Kaolin instead of Lloyd's reagent was used in the purification.

‡ Prepared from Preparation 4 by lead acetate precipitation.

are given in Table I. Table I also contains the analytical data of a number of other preparations, some of which were purified by methods differing slightly from the example cited above. Sulfate could not be detected in any of these preparations. They all show a low uronic acid content⁷ and are nearly neutral with the

⁷ It should be noted that most of the samples contain no uronic acid, within the limits of experimental error. In the method used, 0.1 cc. of 0.01 N acid used for back titration is equivalent to 0.194 mg. of hexuronic acid; i.e., in most of the samples taken, 1.9 per cent. It has been stated (7) that the method is not applicable in the presence of protein. This is

exception of Preparations 1 and 2, which were prepared without gelatin precipitation. The figures for carboxyl merely indicate the approximate acidity, because the buffering capacity of the nitrogenous impurities is undetermined. In some instances a small amount of acetic acid, used for precipitation, may have remained in the preparations.⁴

In Preparation 6, nitrogen was estimated both by the Kjeldahl method and by a micro-Dumas determination.⁸ The Kjeldahl determination, carried out in duplicate, gave a considerably lower value (3.9 per cent) than the Dumas (4.9 per cent), despite the use of 30 per cent H_2O_2 to aid the oxidation (glucosamine hydrochloride also gave too low a Kjeldahl value (6)). The high acetyl value is explained by the presence of traces of sodium acetate used to obtain flocculation from the acetone solution. The yield of the material (67 mg.) did not allow further work with it.

In Preparation 7, ammoniacal acetone (2 per cent NH_4OH in 50 per cent acetone) was used to extract the polysaccharide, giving a much higher yield. The material, however, contained a considerable amount of ammonium salt, which was estimated by distilling a sample with borate. The ammonia nitrogen (2.3 per cent) was subtracted from the total nitrogen figure to give the true nitrogen. Control experiments with other preparations showed that no ammonia was liberated by borate in the absence of ammonium salt. Preparation 7, however, was less active in the blood group test than other preparations.

The amino sugar of Preparation 2 was isolated as the hydrochloride by hydrolyzing 1.8 gm. in the presence of 1.0 gm. of $SnCl_2$ with 65 cc. of 4 N HCl for 8 hours in a boiling water bath. After removal of tin as sulfide and concentration of the solution

not the case in our experience, at least in the absence of interfering substances which would be decarboxylated under the conditions of the reaction. Two examples of recovery may illustrate this point: 4.16 mg. of pure glucuronic acid ($[\alpha]_D^{25}$, +16.05°; m.p., 165°) plus 12.91 mg. of edestin corresponded to 4.30 cc. (corrected for blank) of 0.01 N acid, which equals 4.17 mg. of glucuronic acid (100.2 per cent recovery). 5.42 mg. of glucuronic acid plus 6.84 mg. of edestin corresponded to 5.60 cc. of 0.01 N acid, which equals 5.44 mg. of glucuronic acid (100.4 per cent recovery).

⁸ These analyses were made by Mr. William Saschek of the Department of Biological Chemistry.

in vacuo, 140 mg., plus 77.1 mg. from the mother liquor, of a non-homogeneous material were obtained after several reprecipitations from the methyl alcoholic solution by acetone. This contained on analysis an average of 86.0 per cent hexosamine hydrochloride. This yield corresponds to 29.8 per cent of the colorimetrically determined amount in the starting material. From this preparation by further recrystallization, 65.8 mg. of pure hydrochloride were obtained with $[\alpha]_D^{24}$ at equilibrium of $+71.8^\circ \pm 1.0^\circ$ ($[\alpha]_D$ of glucosamine hydrochloride, $+72.5^\circ$), equivalent weight (in the presence of formaldehyde) 217 (theoretical, 215.6). The mother liquor, on concentration, yielded more crystalline material, which, however, was not isolated since it was obviously glucosamine hydrochloride.

Galactose was identified as the other component of the polysaccharide by oxidation with nitric acid according to van der Haar (8). The formation of mucic acid is proof here of galactose, since uronic acid, for all practical purposes, is absent. On two different samples of low uronic acid content, a quantitative mucic acid determination (8) was carried out. In the first case, 0.1428 gm. of mucic acid was isolated from 1.000 gm. of the sample, corresponding to 0.229 gm., or (corrected) 26.1 per cent galactose. The mucic acid was identified by its crystalline form, by the preparation of its typical thallium salt, and by its melting point: m.p., 206–207°; m.p. of authentic mucic acid, 207°; mixed m.p., 207–208°. In the second case, 0.1771 gm. of mucic acid was obtained from 1.000 gm. of Preparation 5 after previous hydrolysis with 5 per cent H_2SO_4 and addition of 0.5000 gm. of sucrose. The mucic acid yield corresponded to 0.265 gm. or (corrected) 28.8 per cent galactose. The glucosamine content of the polysaccharide was 38.6 per cent. These percentages are in accord with an equimolar ratio of glucosamine and galactose, when allowance is made for the low values usually obtained in the mucic acid procedure.

Preparation of Acid Polysaccharide—The preparation of the acid polysaccharide from the gelatin salt was attempted. The material obtained after solution of the gelatin salt in alkaline water, removal of the gelatin with chloroform and amyl alcohol, and precipitation by glacial acetic acid on analysis was always found to be a mucoid; *e.g.*, N, 13.6 per cent; hexosamine, 10.7 per cent.

Evidently the polysaccharide is quite firmly held by the protein. Alkaline extraction, therefore, was resorted to for the preparation of the acid fraction.

A 40 gm. portion of the original commercial sample was extracted for 2 days at room temperature with 400 cc. of N NaOH, and the supernatant solution was precipitated by 2 volumes of alcohol. The precipitate was washed with alcohol, dissolved in a small amount of H_2O , and reprecipitated by 10 volumes of glacial acetic

TABLE II
Analyses of Acid Polysaccharide

Analysis	Preparation A-1		Preparation A-2	
	per cent	equivalents per equivalent weight	per cent	equivalents per equivalent weight
Nitrogen.....	4.0	1.9	3.0	1.4
Glucosamine.....	32.8	1.2	30.6	1.2
Uronic acid.....	39.1	1.3	27.6	1.0
Acetyl.....	8.8	1.4	8.8	1.4
Sulfur (see foot-note 8).....	4.7*	1.0	3.1†	0.6
Equivalent weight.....	659		672	
	per cent		per cent	
Moisture.....	5.9		2.3	
Ash.....	8.2		9.7	
	degrees		degrees	
$[\alpha]_D^{25}$	-20.2		-22.2	
$[\alpha]_D^{25}$ (neutralized).....	-35.7			

* Determined as acid-hydrolyzable sulfate.

† Determined as total sulfate, found to be distributed equally in the residue and volatile portion.

acid. This precipitate was washed free from acetic acid with alcohol and acetone, and dried. The product (about 3 gm.) was dissolved in 150 cc. of H_2O with neutralization; the solution was brought to 0.1 N HCl and shaken for a short time with 15 gm. of Lloyd's reagent. After separation of the supernatant fluid by centrifugation, the precipitate was washed once with 50 cc. of H_2O , and the combined supernatant solutions were precipitated by 4 volumes of alcohol. The precipitate was washed with alcohol and twice extracted with 25 cc. of H_2O . The aqueous extracts were precipitated by 5 volumes of alcohol, washed, and dried. The precipi-

tate consisted of 1.09 gm. of a white powder (Preparation A-1). In a similar experiment, 50 gm. were extracted for 4 days with 500 cc. of 0.5 N NaOH. After precipitation of the acid in the manner described above, it was converted first into the neutral lead salt, and then into the acid barium salt. A yield of 1.827 gm. was obtained (Preparation A-2). The analyses of these substances appear in Table II.

From 0.877 gm. of Preparation A-1, 0.1833 gm. of hexosamine hydrochloride was isolated in the first fraction, and 0.0936 gm. in the second fraction. It was not recrystallized. This corresponded to 93 per cent of the colorimetrically determined hexosamine. At equilibrium $[\alpha]_D^{25}$ was $+73.2^\circ$; equivalent weight found (formol titration) 216.5, calculated 215.6. The amino sugar from Preparation A-2 had at equilibrium $[\alpha]_D^{25}$ of $+71.9^\circ$; equivalent weight found, 214. Thus the sugar was glucosamine.

DISCUSSION

In our studies on tissue polysaccharides we have attempted to isolate and characterize the sugars as far as possible without the use of strong reagents. The isolation of such polysaccharides is apparently not very difficult if they are acid, but difficult if the sugar is non-acidic. So far, examples of two such non-acidic amino sugar-containing polysaccharides from animal sources have been reported in the literature, the polysaccharide from egg white (9, 10) and from serum (11). In both instances a glucosamine dimannoside was obtained. To these two possibly identical sugars, a third may now be added, the glucosamine galactoside from the gastric mucosa of the pig. This third sugar was obtained without the use of strong alkali, whereas the other two were obtained by boiling with 10 per cent Ba(OH)₂ for several days. Assuming an equimolar ratio of acetylglucosamine and galactose, our preparations have a purity of about 75 per cent on the basis of the glucosamine values. Since most of the preparations give a positive Ehrlich's diazo reaction, it may be concluded that histidine is still present. According to Rimington (11) it is also difficult to remove the histidine from the glucosamine dimannoside of serum. In both instances apparently the sugar in the native state is firmly bound to a polypeptide chain, which is partly broken either by alkaline hydrolysis (Rimington) or by the action

of pepsin in our case. With the neutral gastric mucin polysaccharide, the use of alkali has to be avoided, since this treatment not only abolishes the blood group reaction, but also greatly decomposes the sugar. This latter fact probably accounts for previous failures to obtain this polysaccharide. In the case of the serum polysaccharide, the destruction due to alkaline hydrolysis is also very great (11).

Some of our typical neutral polysaccharide preparations were tested for the blood Group A factor by Dr. K. Landsteiner,⁹ who intends to report in detail later.¹⁰ The Group A reaction was demonstrated serologically with quantities of the order of 5 to 10×10^{-10} gm. In the authors' opinion, the polysaccharide itself seems to be responsible for the reaction. Glucosamine and galactose have been found also in the blood Group A factor isolated from human urine (13). This preparation is apparently contaminated with an acid fraction.¹¹ Whether the blood Group A factors from all sources are chemically identical is unknown at present.

A polysaccharide of similar composition was recently isolated and characterized by Morgan (15) as the soluble specific substance of the Shiga-Kruse bacillus. In contrast to our polysaccharide, it contains 1 mole of glucosamine per 2 moles of galactose. That the two polysaccharides are not identical is shown by the failure of the gastric sugar to react with the Shiga bacillus antiserum.¹² It is noteworthy that this polysaccharide, in contrast to ours, was obtained apparently free of amino acids, despite the fact that we used to a certain extent the same fractionation scheme that Morgan used.

⁹ We wish to take this opportunity to thank Dr. K. Landsteiner for these tests, and also for much valuable information.

¹⁰ While this work was in progress, an apparently similar material was found by Landsteiner and Chase (12) in commercial pepsin. We have also obtained the same polysaccharide from this source, where it is present in considerable quantity.

¹¹ In a recent preliminary note, Freudenberg and his associates (14) have announced that they have obtained from cattle and pig organs very active Group A preparations, containing 5.8 per cent of nitrogen and 6.5 per cent of acetyl. The preparations had an acid reaction and gave a negative test for hexosamine.

¹² This test was carried out by Dr. Richard Thompson of this department.

Also in the isolation of other neutral bacterial polysaccharides (16, 17) no great difficulty is encountered in obtaining them pure. Either these sugars are free in the bacterial membrane, or bacterial enzymes during the isolation hydrolyze a linkage between the sugar and a peptide.

The acid polysaccharide containing glucosamine and sulfuric acid is present in much lower concentration. The specific rotation of this acid ($[\alpha]_D^{25}$ of the acid, -20.2° , -22.2° ; of the neutral sodium salt, -35.7°) is very similar to that of the chondroitin-sulfuric acid isolated in this laboratory ($[\alpha]_D^{25}$ of the acid, -20.1° ; of the neutral sodium salt, -37.1°). It also resembles chondroitin-sulfuric acid in the stability of its protein salts.

SUMMARY

The occurrence of two polysaccharides has been demonstrated in a commercial sample of pig gastric mucin, the one a neutral polysaccharide consisting of acetylglucosamine and galactose in apparently equimolar ratio, the other an acid polysaccharide containing acetylglucosamine, hexuronic acid, and ester sulfate. The neutral polysaccharide, obtained free of the acid sugar, gives a blood Group A reaction in quantities of 5 to 10×10^{-10} gm. It is present in the material in much higher concentration than the acid sugar, and is apparently responsible for the highly viscous nature of gastric mucin.

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THE OXIDATION OF HEXOSAMINES: *d*-GLUCOSAMINE AND *d*-GLUCOSAMINIC ACID

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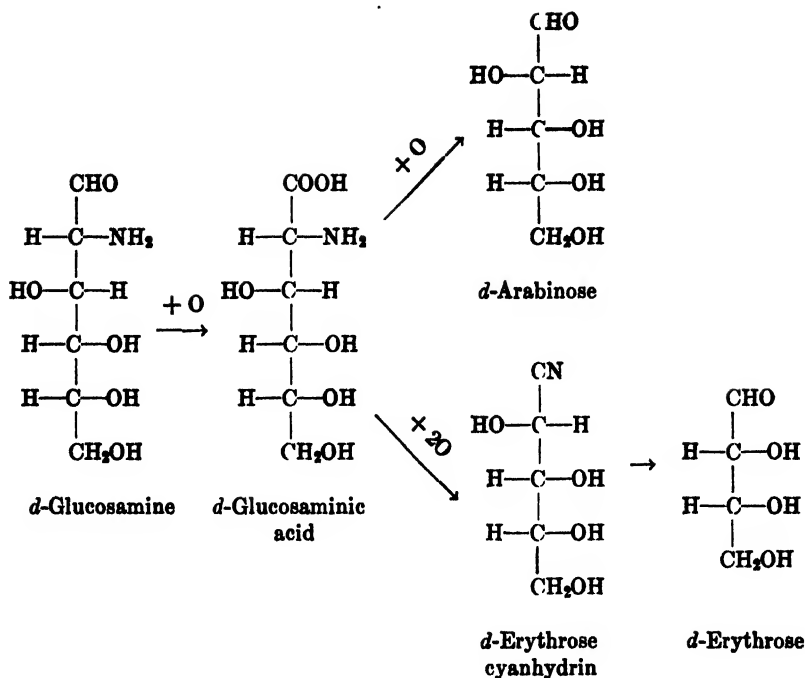
The physiological and chemical relationships of the hexosamines to other compounds have been little studied, although their rather wide-spread occurrence in the animal body is suggestive of their importance in biological systems. They may conceivably be precursors of pentoses, the source of which in the animal body is still in doubt in spite of their frequent occurrence. For example, *d*-ribose is widely distributed as a component of the nucleotides, and *l*-xyloketose is occasionally encountered in cases of pentosuria. Experiments reported by Enklewitz and Lasker (1) indicate that *d*-glucuronic acid causes excretion of increased amounts of *l*-xyloketose in pentosurics; however, the manner in which the pentose could be formed from glucuronic acid is quite obscure, and the formal relationship between the two substances does not suggest a simple transformation. Another form of pentosuria in which *dl*-arabinose appears is extremely rare.

The experiments to be reported in this paper were undertaken in order to realize *in vitro* some of the possible relationships between hexosamines and pentoses. A search of the literature revealed little information regarding the oxidation products of *d*-glucosamine other than glucosaminic acid. Neuberg (2) reported the formation of pentoses during the oxidation of glucosaminic acid with Fenton's reagent, but failed to characterize the products. More recently Zechmeister and Tóth (3) showed that small amounts of a pentose giving the phenylosazone of *d*-arabinose are formed by treating *d*-glucosamine with nitrous acid. The principal product of the reaction was however a hexose yielding phenylglucosazone.

Successful oxidation of hexosamines to pentoses depends primarily upon the choice of oxidizing agent. For this purpose chloramine-T (*p*-toluene-sodium-sulfonchloramide) seemed eminently suited. Dakin (4) demonstrated the usefulness of this reagent in the oxidation of amino acids, in which case aldehydes are formed with very little further oxidation. The analogy to the case in hand is particularly evident in the oxidation of β -hydroxyglutamic acid (5) in which the semialdehyde of malic acid is formed, and α -amino- β -hydroxybutyric acid (6) in which the aldehyde of lactic acid is formed. Furthermore, Engfeldt (7) has shown that under the same conditions sodium hypochlorite easily oxidizes glucose, while chloramine-T causes practically no oxidation.

Preliminary experiments indicated that *d*-glucosaminic acid could be oxidized easily by chloramine-T, and that the reaction mixture contained pentoses. In subsequent experiments it was possible to isolate *d*-arabinose in yields of 40 to 50 per cent as well as small amounts of *d*-erythrose in the form of their benzylphenylhydrazones. The same products were obtained from *d*-glucosamine under similar conditions, in which case oxidation to glucosaminic acid may be considered as the first step in the reaction. Under the same mild conditions acetyl-*d*-glucosamine showed no tendency to become oxidized. The isolation of *d*-erythrose is interesting, since it shows that the oxidation proceeded along two paths. It will be recalled that Dakin (8) demonstrated that oxidation of amino acids with 2 equivalents of chloramine-T instead of 1 resulted in the formation of a nitrile in place of the aldehyde. Since hydrocyanic acid was also found as a reaction product, the erythrose would seem to be a secondary product formed by the elimination of hydrocyanic acid from its cyanhydrin. The reactions involved are formulated below.

The ease of oxidation of glucosamine may justify the suggestion that it should be considered as a possible source of related pentoses in biological systems. In this connection it may be recalled that *d*-arabinose bears the same structural relationship to *d*-ribose as *d*-mannose does to *d*-glucose. Experiments with other amino sugars and other oxidizing agents are in progress and will be reported in the near future.



EXPERIMENTAL

Oxidation of d-Glucosaminic Acid—*d*-Glucosaminic acid was prepared from *d*-glucosamine by the method of Pringsheim and Ruschmann (9). A solution of 0.98 gm. (5 mm) of glucosaminic acid in 25 cc. of water and 5 cc. of normal sodium hydroxide was placed in the incubator at 37.5°. At hourly intervals 5 cc. portions of an aqueous solution of chloramine-T, 0.31 gm. (1.1 mm) per 5 cc., were added until a total of 25 cc. was reached. The resulting mixture was allowed to remain in the incubator for 6 hours after the last addition of chloramine-T,¹ when it was treated with 5 cc. of normal hydrochloric acid, chilled quickly in an ice bath, and filtered to remove the toluenesulfonamide which had precipitated during the reaction. The filtrate was evaporated to dryness in a

¹ The oxidation may be carried out with only slightly decreased yield of *d*-arabinose by adding the whole of the oxidizing agent immediately. In this case the reaction mixture should be left in the incubator for 10 to 12 hours.

vacuum at 30–40°; the residue was extracted with ice-cold water, leaving a further small quantity of toluenesulfonamide undissolved, and the clear supernatant liquid was again evaporated to dryness as above. The residue was now taken up in a small volume (12 to 15 cc.) of 50 per cent alcohol, and the resulting solution was heated to boiling and treated with 1.2 gm. of benzylphenylhydrazine suspended in 3 cc. of 90 per cent alcohol. On standing 24 to 36 hours at 20–25°, and finally in the ice chest for some hours, a copious precipitate of *d*-arabinose benzylphenylhydrazone formed. The hydrazone was filtered off by suction and was practically pure after washing on the filter with cold 50 per cent alcohol and with ether; yield 40 to 45 per cent of the theory, m.p. 172–173° (corrected) with decomposition. One or two recrystallizations from 75 to 95 per cent alcohol led to analytically pure material, m.p. 173–173.5° (corrected) with decomposition, showing no depression of the melting point when mixed with an authentic specimen.

*Analysis*²—C₁₁H₁₁N₂O₄. Calculated. C 65.41, H 6.72, N 8.48

Found. " 65.43, " 6.60, " 8.58

*Rotation*³— $[\alpha]_D^{25} = +11.7^\circ$ (0.4% in absolute methyl alcohol) .

The filtrate from the arabinose benzylphenylhydrazone usually smelled of hydrocyanic acid; in fact sufficient of the substance was present in the vapor over the solution to cause definite turbidity in a drop of silver nitrate solution exposed to the vapor. When it was further found that the carbohydrate mixture obtained by decomposing the residual hydrazones in the filtrate with formaldehyde⁴

² The elementary microanalyses reported in this paper were carried out by Mr. William Saschek of this Department.

³ The specific rotations of specimens of *d*-arabinose benzylphenylhydrazone obtained from glucosaminic acid, glucosamine, and an authentic specimen of *d*-arabinose all agreed with the values $[\alpha]_D = +11.4^\circ$, $+11.9^\circ$ respectively) given by Chargaff and Anderson (10) and Votoček, Valentin, and Leminger (11). These values are lower than those given in older papers ($[\alpha]_D = +14.4^\circ$), but in this respect it should be mentioned that it was impossible to prepare solutions more concentrated than 0.4 per cent, although concentrations of 0.5 per cent and over are employed in the older reports (Ruff and Ollendorff (12) and Fischer, Bergmann, and Schotte (13)). Small amounts of impurities markedly increase the solubility of this derivative.

⁴ Crystalline *d*-erythrose benzylphenylhydrazone was first obtained by treating the absolute alcoholic solution of this carbohydrate mixture with

would reduce Benedict's solution slowly in the cold, the presence of a tetrose was suspected. *d*-Erythrose benzylphenylhydrazone was isolated from the filtrate by evaporation to a syrup in a vacuum at 30°, and extraction of the residue with boiling benzene. The benzene solution first deposited several small fractions of the arabinose derivative, and then, on treatment with petroleum ether (60–80°), small quantities of *d*-erythrose benzylphenylhydrazone. Crystallization of the latter from the crude mixture was very slow and was induced only with difficulty even after material for seeding became available. Purification was readily accomplished by several recrystallizations from benzene and benzene-petroleum ether (1:1 by volume). About 25 mg. of pure material were obtained, ^s m.p. 105–105.5° (corrected).

Analysis—C₁₇H₁₈N₂O₅. Calculated. C 67.96, H 6.72, N 9.33
Found. " 67.91, " 6.57, " 9.31

Rotation—[α]_D²⁰ = –29° (0.7% in 95% ethyl alcohol)

Ruff (14) reports the specific rotation of *d*-erythrose benzylphenylhydrazone as –32°, and the melting point as 105.5° (corrected).

In a similar experiment in which 0.98 gm. of glucosaminic acid was oxidized with 2 equivalents of chloramine-T, the yield of *d*-arabinose dropped to 20 per cent. A somewhat larger amount of the erythrose derivative having the constants reported above was obtained.

Oxidation of d-Glucosamine—The oxidation of *d*-glucosamine was carried out in essentially the same manner as described for glucosaminic acid. Glucosamine hydrochloride (5 mm) was

benzylphenylhydrazine, evaporating to dryness in a vacuum desiccator, and extracting the residue with boiling benzene. Careful treatment of the benzene solution with petroleum ether gave first several small fractions of the arabinose derivative, and then on long standing with frequent scratching small amounts of the erythrose derivative were induced to crystallize. In agreement with the findings of Ruff (14) it was found that the use of benzaldehyde to decompose the hydrazone of erythrose seems to cause considerable decomposition of the sugar.

^s The isolation of *d*-erythrose benzylphenylhydrazone must be considered as qualitative rather than quantitative owing to the reluctance with which it crystallized from the mixture of accompanying reaction products.

dissolved in water and treated with 1 equivalent of sodium hydroxide. 2 equivalents of chloramine-T dissolved in water were added either at once or in portions as described above. The concentration of the chloramine-T solution was twice that used above so as to maintain the same concentration with respect to the amino sugar. The total reaction time was 8 to 10 hours in all experiments. The addition of hydrochloric acid at the end of the reaction period was omitted, but the isolation of *d*-arabinose and *d*-erythrose was accomplished as already described. Portionwise addition of the oxidizing agent permitted the isolation of arabinose in yields of 40 per cent, while addition of the entire amount at the start caused the yield to drop to about 20 per cent.

The crude *d*-arabinose benzylphenylhydrazone melted at 170–171° (corrected), with decomposition; after one or two recrystallizations from 75 to 95 per cent alcohol the melting point was 172.5–173° (corrected), with decomposition, and showed no depression when mixed with an authentic specimen.

Analysis— $C_{11}H_{13}N_2O_4$. Calculated. C 65.41, H 6.72, N 8.48

Found. " 65.53, " 6.66, " 8.51

Rotation— $[\alpha]_D^{25} = +11.4^\circ$ (0.4% in absolute methyl alcohol)

From 1.08 gm. (5 mm) of glucosamine hydrochloride about 30 mg. of *d*-erythrose benzylphenylhydrazone were obtained, m.p. 105–106° (corrected).

Analysis— $C_{17}H_{19}N_3O_3$. Calculated. C 67.96, H 6.72, N 9.33

Found. " 68.18, " 6.68, " 9.32

Rotation— $[\alpha]_D^{25} = -28.2^\circ$ (1.5% in 95% alcohol)

Oxidation of Acetyl-d-Glucosamine—Acetyl-*d*-glucosamine was prepared by the method of Zuckerkandl and Messiner-Klebermass (15). A solution of 5 mm of the substance and 5.5 mm of chloramine-T both with and without the addition of 1 equivalent of sodium hydroxide was kept at 37.5° for 48 hours, but showed no evidence of oxidation. The possibility of oxidation under more vigorous conditions is being investigated.

SUMMARY

The oxidation of *d*-glucosamine and glucosaminic acid with chloramine-T in aqueous solution has been studied. Both sub-

stances were easily oxidized at 37.5°, yielding *d*-arabinose as the principal product together with small amounts of *d*-erythrose cyanhydrin. Acetyl-*d*-glucosamine is not oxidized under these conditions.

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THE DETERMINATION OF BROMINE IN BIOLOGICAL SUBSTANCES*

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Zondek and Bier ((1932) p. 633; (1933)) reported that the normal bromine content of the blood was greatly diminished during certain stages of manic-depressive psychoses. They also reported ((1932) p. 759) that the hypophyseal bromine diminished or disappeared during artificial sleep, whereas the bromine content of the mesencephalon increased. The authors suggested that the pituitary secreted a substance containing bromine which could act upon the cerebral centers producing sleep. These papers aroused a great deal of interest in the subject of the physiological rôle of bromine. The work has been repeated and extended by other investigators, some substantiating it (see Urechia and Retezeanu (1935)) but the majority being unable to confirm the results (see Dixon (1935)).

The method of Roman (1929) and Pincussen (1932) employed by Zondek and coworkers has been the subject of considerable study and is known to be subject to gross errors. During the last few years a number of new micromethods for the estimation of bromine have been described (see Olszycka (1935) and Neufeld (1936)). The procedure of Dixon (1934) has been carefully examined by the present authors and, with certain modifications, has been found to give satisfactory results. Briefly, his method is as follows: The organic material is destroyed by heating with alkali in a nickel crucible and the bromide and other soluble salts are extracted with hot water and then filtered. The filtrate is

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evaporated and heated again to destroy any residual organic matter and the bromide is extracted from the residue with 90 to 95 per cent alcohol. The bromide is oxidized to bromate by hypochlorite in the presence of borate buffer and half saturation with sodium chloride according to the equation $3\text{KClO} + \text{KBr} \rightarrow \text{KBrO}_3 + 3\text{KCl}$. The bromate formed is determined iodometrically according to the equation $\text{HBrO}_3 + 6\text{HI} \rightarrow \text{HBr} + 3\text{H}_2\text{O} + 3\text{I}_2$. By this procedure the amount of iodine liberated is equivalent to 6 times the original amount of bromine present.

It was found that the recovery of known amounts of bromide was low when the procedure of Dixon was carefully followed. The low results could be due to (1) incomplete oxidation of bromide to bromate, (2) incomplete extraction of the bromide by the alcohol, (3) incomplete extraction of the bromide from the residue of the ashing procedure, (4) loss of bromide during ashing, (5) incomplete destruction of or contamination with organic material before the oxidation of bromide to bromate. Each of these possibilities has been investigated and certain modifications to Dixon's original method have been made which permit quantitative recovery of known amounts of bromide. Another disadvantage of the original method was the long time required to carry out a determination. By use of the overhead heater described by Nims and Horwitt (1936) for preliminary ashing and evaporations, the time required has been greatly shortened.

Experiments were carried out to determine whether bromide is quantitatively oxidized to bromate under the conditions specified by Dixon. The results are recorded in Table I and indicate that the oxidation is quantitative. Blanks on the reagents were carried out and found to be very constant for any given preparation of KClO and are probably due to the presence of a small amount of KClO_3 unavoidably formed in the preparation of the KClO . The results of the blanks of three different preparations of KClO are also given in Table I.

Investigation of the extraction of bromide with alcohol showed that quantitative recovery of known amounts of bromide was seldom obtained. By increasing the amount of alcohol used and the number of extractions the results were not improved. The presence of any organic material in the reaction mixture at the time of oxidation of the bromide to bromate will lead to low results

and this was suspected as the cause of the poor recoveries. The organic material may be present because of incomplete destruction during the ashing, contamination from the environment during the extraction, or formation of traces of non-volatile organic matter from the alcohol by atmospheric oxidation during evaporation. It was found that if the flask containing the residue after the evaporation of the alcohol was heated at 480° for 2 minutes, all traces of organic matter were destroyed and quantitative recoveries of bromide were obtained. The results are summarized in Table II.

TABLE I .

Oxidation of Bromide to Bromate by Hypochlorite

Bromide added, 50.0 micrograms.

Hypochlorite preparation No.	Blank on reagents, 0.002 N $\text{Na}_2\text{S}_2\text{O}_8$	Bromide determined by titration
	ml.	micrograms
1	0.120	50.2
1	0.130	49.8
1	0.125	49.1
1	0.115	50.8
2	0.240	50.8
2	0.225	50.0
2	0.250	48.6
2	0.240	50.8
3	0.210	49.7
3	0.220	50.0
3	0.210	50.2
3	0.185	50.6

Modification of the ashing procedure by use of the overhead heater has proved very advantageous in the matter of time saved and in the prevention of loss of bromine due to excessive smoking and swelling during the preliminary heating of the material. Six or eight samples can be analyzed at the same time and the whole procedure completed in 2 days. By ashing one group of determinations while carrying out the alcoholic extraction of another group, it is possible to complete six or eight determinations every day. This is about one-fifth the time required when an overhead heater is not used. It was found that 0.1 N K_2CO_3 is better to use for extracting the bromide from the ash, because

substances insoluble in alkali are therefore prevented from being deposited in the filter paper. When water is used, this does occur and much time is lost during the filtering process.

Reagents Required—

Water distilled from alkali in an all-glass apparatus.

5 N KOH solution prepared free from halogens by the electrolytic method described by Harvey (1935).

5 N K_2CO_3 solution prepared by passing pure CO_2 into a solution of halogen-free KOH.

TABLE II
Extraction of Bromide with Alcohol

Volume of alcohol used	No. of extractions	Heated to 490° after extraction	Bromide added	Bromide recovered
ml.			micrograms	micrograms
30	4	Not heated	50.0	43.0
30	4	" "	50.0	40.1
40	6	" "	50.0	40.0
40	6	" "	50.0	46.6
40	6	" "	50.0	39.4
40	6	Heated	50.0	48.4
40	6	"	50.0	49.5
40	6	"	50.0	52.0
40	6	"	25.0	24.8
40	6	"	25.0	24.2
40	6	"	12.5	12.5
40	6	"	12.5	12.3
40	6	"	5.0	4.8
40	6	"	5.0	4.5

0.1 N K_2CO_3 solution prepared by diluting the halogen-free 5 N K_2CO_3 solution.

Absolute ethyl alcohol redistilled in an all-glass apparatus from solid K_2CO_3 .

1 N $KClO_3$ solution in 0.1 N KOH; made by slowly passing 9 gm. of chlorine gas into 225 ml. of 1.25 N halogen-free KOH and finally making up to 250 ml. with water. The KOH is contained in a flask cooled in ice while the chlorine is passing through to minimize formation of $KClO_4$.

Saturated NaCl solution. The NaCl is freed of bromide by dissolving the salt in water and precipitating it with redistilled

alcohol. By repeating the procedure two or three times the NaCl is completely freed from bromide.

Saturated boric acid solution. The boric acid is purified by recrystallization twice from water.

1 N KI solution made up fresh just before use.

5 N HCl solution.

0.5 per cent starch solution made up fresh just before use.

1 N H_2O_2 solution.

0.002 N $\text{Na}_2\text{S}_2\text{O}_3$ solution made up from a stock 0.02 N $\text{Na}_2\text{S}_2\text{O}_3$ solution and standardized just before use.

For the analysis of blood, 2 to 10 gm. of whole blood are weighed in a weighing bottle and transferred to a nickel crucible of 8 cm. diameter; 25 ml. of redistilled water are used to wash the blood into the crucible quantitatively. 0.5 gm. of halogen-free sucrose, 2 ml. of 5 N KOH, and 2 ml. of 5 N K_2CO_3 are added and the mixture evaporated and charred by heating with an overhead heater. The rate of evaporation and charring necessary to prevent spattering, swelling, and vigorous smoking is easily regulated by adjusting the distance between the heater and the crucible. When the tendency to swell has subsided, the crucible is put in an electric muffle furnace at 300° , and the temperature rapidly increased to 480° and held there for half an hour. The crucible is then removed and cooled, and its contents are ground up with a small amount of water by means of a glass rod. The mixture is evaporated to dryness with the overhead heater and heated in the muffle furnace at 480° for half an hour. The grinding with water, evaporating, and heating for half an hour at 480° are repeated two more times. 20 ml. of water are then added to the crucible, which is heated for a few minutes on the water bath. The contents are triturated with a glass rod and then filtered through an 11 cm. Whatman No. 50 filter paper (previously well washed with hot water) into an 8 cm. porcelain dish. The residue in the crucible is extracted five more times, with 10 ml. of 0.1 N K_2CO_3 for each extraction. The combined filtrate in the porcelain dish is evaporated to dryness and then ignited in the muffle furnace for half an hour at 480° . The contents are dissolved in 15 ml. of water and filtered through a 9 cm. Whatman No. 42 filter paper into another porcelain dish, being washed five times with 3 ml. of 0.1 N K_2CO_3 to insure a quantitative transfer. The solution is

evaporated and the residue ignited in the muffle furnace for 5 minutes at 480° .

The alkaline mass is rubbed up to a smooth cream with sufficient water (about 2.0 ml.) to produce an almost complete solution. Absolute ethyl alcohol is added slowly from a burette with continuous stirring with a round ended glass rod. After the addition of 5 ml. of alcohol, the mixture is vigorously stirred for 1 minute. This procedure is repeated until 15 ml. of alcohol have been added. The mixture is rubbed up until the potassium carbonate passes into a thin "pasty" condition and then the alcoholic layer is decanted through a Jena glass sintered filter No. 3 into a 50 ml. Erlenmeyer flask. The residue is again extracted with 5 ml. of alcohol. To the alkaline mass from which the alcohol has been drained 0.6 ml. of water is added and the alcoholic extraction is again carried out. This is repeated until 40 ml. of alcohol in all have been used. The contents of the flask, with addition of a few pumice granules to facilitate smooth boiling, are evaporated to dryness with the overhead heater. The last traces of organic material are destroyed by heating in the muffle furnace at 480° for 2 minutes. To the cooled flask are added 2 ml. of water, 1 ml. of KClO_4 , and 2.5 ml. of saturated NaCl ; the mixture is warmed to 85° on a water bath and treated with 1 ml. of saturated H_3BO_3 . The flask is removed after 20 minutes, cooled, and 1 ml. of 1 N H_2O_2 added, after which the contents are gently boiled for 8 minutes. The flask is cooled and 0.3 ml. of 5 N HCl , 3 drops of 0.5 per cent starch, and 1 ml. of 1 N KI are added, after which the solution is titrated against 0.002 N $\text{Na}_2\text{S}_2\text{O}_3$ with a 10 ml. microburette. 1 ml. of 0.002 N $\text{Na}_2\text{S}_2\text{O}_3$ = 26.7 micrograms of Br.

The $\text{Na}_2\text{S}_2\text{O}_3$ is standardized by using 2 ml. of a stock KBr solution containing 50.0 micrograms of bromide and oxidizing it to bromate in exactly the same manner as the sample being analyzed. A blank determination must be carried out at the same time and under identical conditions as for the substance being analyzed in order to correct for traces of bromine that may be present in the reagents, filter papers, etc., and to insure that bromine is not being introduced from the atmosphere of the laboratory or other sources. Great care must be taken to keep the atmosphere of the room in which the analytical work is being done free of bromine. This is sometimes difficult if bromine or bromine water is being used as a reagent in the same building.

It has been found that the method is applicable to biological materials in general. For organic substances at least 1.0 ml. of 5 N alkali should be used for every 1 gm. quantity of dry organic matter to be ashed. Fatty materials must be saponified in the cold with alcoholic potassium hydroxide before ignition. The substance is placed in an Erlenmeyer flask and 25 ml. of re-distilled 95 per cent alcohol, 0.5 ml. of 5 N KOH, and 0.5 ml. of 5 N K_2CO_3 are added for each gm. of sample, and the mixture is allowed to stand for several days with occasional shaking. The contents of the flask are then transferred to a nickel crucible and treated as previously described.

TABLE III
Recovery of Added Bromine

Material analysed	Bromine found per gm. sample	Bromine added per gm. sample	Total bromine		Difference
			Estimated	Found	
	<i>micro-grams</i>	<i>micro-grams</i>	<i>micro-grams</i>	<i>micro-grams</i>	<i>micro-grams</i>
Calf Meal (Maynard, 1930)...	16.5	25 0	41.5	40.4	-1.1
Sucrose.....	None	100.0	100.0	96 8	-3.2
"	"	25.0	25.0	25.0	0
"	"	50.0	50.0	49.2	-0.8
Crisco.....	"	5.0	5.0	4.4	-0.6
Casein.....	1.4	10.0	11.4	11.8	+0.4
Yeast.....	4.2	10.0	14.2	14.5	+0.3
Rat blood.....	13.5	12.5	26.0	26.8	+0.8
Wheat germ extract.....	8 9	10 0	18.9	18.3	-0.6
Synthetic rat diet.....	20.2	50.0	70 2	71.2	+1.0

Iodides are also quantitatively oxidized by hypochlorite to iodates and are therefore estimated together with bromine. Normally, animal and plant tissues and fluids contain very small amounts of iodine compared to bromine and no appreciable error is introduced in analyses by disregarding the iodine. However, in certain substances such as thyroid gland, blood after iodine therapy, and marine plants and animals, it is necessary to determine the iodine separately and deduct it from the total iodine and bromine figure.

In Table III results of the analyses of various substances are given and the recovery of known added amounts of bromide. The

TABLE IV

Bromine Content of Various Foodstuffs

Micrograms of bromine per gm. of material.

Casein	
Commercial.....	8.1
Vitamin-free (Labco brand).....	2.1
Salt-low (washed with distilled water).....	2.4- 2.9
" (" " redistilled water and redistilled alcohol).....	1.3- 1.6
Salt-low (reprecipitated three times).....	1.4
" (" " in presence of alcohol).....	1.3- 1.4
Lactalbumin (extracted with hot alcohol).....	5.1
Edestin.....	2.9
Egg albumin... ..	94.0
Milk powder (Parlac brand).....	40.0-42.0
Whey "	17.3
White flour (Gold Medal).....	7.9
" " (Swans Down).....	5.2
Yellow corn-meal.....	3.4
Sucrose.....	0
Dextrin.....	1.8
Fat (Pancrust).. ..	0 - 1.0
" (Crisco).....	0 - 0.5
Wheat germ oil.....	0.5- 2.0
Halibut liver oil with viosterol.....	10 0
Alcoholic extract of wheat germ	8.7- 8.9
Lilly's No. 343 liver extract.....	800
Galen B.....	33.0
Concentrate of Galen B.....	78.0
Northwestern yeast.....	3.1- 4.0
Milbrew yeast.....	2.6- 4.6
" " concentrate.....	19 3
Harris " " 	13.0
Anheuser-Busch yeast (Strain L).....	8.3
" " (" S).....	8.8
" " concentrate.....	13.6
Vitamin B concentrate (Vitab).....	16.7
Pabst yeast.....	3.5
" " (debittered).....	1.0- 1.8
Kayson's A-1 Calf Meal.....	16.5-20.2

method has been used to analyze a large number of food products; the results are recorded in Table IV.

SUMMARY

The method of Dixon for the estimation of bromine in biological materials has been studied and certain modifications have been introduced; accurate results are obtained and the time required for completion of the analysis is decreased.

With the method outlined a number of foods have been examined for bromine content.

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SYNTHESIS OF α -AMINO- β -HYDROXY-*n*-BUTYRIC ACIDS

III. A SIMPLE METHOD OF PREPARING A MIXTURE OF THE TWO FORMS

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As was reported in a previous article (1), it seemed probable that *dl*-threonine,¹ uncontaminated by its diastereomer, could not be prepared from the mercuration product of ethyl crotonate. Therefore, we began an investigation of other methods which we hoped would yield mainly threonine.

The first reaction which was studied was the bromination of crotonic acid in methyl alcohol solution. An excellent yield of α -bromo- β -methoxy-*n*-butyric acid-A² was obtained. Unfortunately it was not the form yielding threonine. This work will be reported in detail later, since pure bromomethoxybutyric acid-A is much more readily prepared by this method than by that previously used (1, 3).

At that time we had just completed a study (4) of the mercuration of cinnamic acid, and the bromination of the α -bromomercuri- β -methoxy- β -phenylpropionic acid obtained from the cinnamic acid-mercuric acetate addition product. Since rather interesting findings were obtained with cinnamic acid, it was decided to investigate the behavior of crotonic acid in analogous reactions. Although pure α -bromo- β -methoxy-*n*-butyric acid-B was not obtained, the results were highly pleasing, since the reactions furnished a simple and rapid method of preparing a mixture of

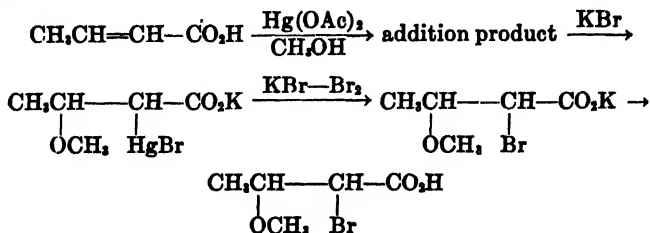
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¹ The naturally occurring α -amino- β -hydroxy-*n*-butyric acid has been named *d*(-)-threonine (Meyer and Rose (2)). *dl*-Threonine is used to designate the racemic mixture of *d*(-)-threonine and its enantiomorph.

² The suffix "A" is used to indicate substances related to the amino-hydroxybutyric acid prepared by Abderhalden's method and the suffix "B" to indicate the precursors of *dl*-threonine.

the bromo acids which contained 30 to 40 per cent of the precursor of threonine. This new method made it possible to synthesize large amounts of mixtures of the aminomethoxybutyric acids and of the aminohydroxybutyric acids with such ease and rapidity that the preparation of threonine by separation of the mixtures became feasible. The methods of separation will be described in the succeeding paper.

The reactions carried out are shown in the accompanying equations.



Crotonic acid in methyl alcohol solution readily added mercuric acetate. The product obtained varied in decomposition point and in mercury content with the length of time of mercuration. From analogy one might expect to obtain anhydro- α -hydroxy-mercuri- β -methoxy-*n*-butyric acid which contains 63.48 per cent of mercury. Another possible product of the reaction is α -acetoxy-mercuri- β -methoxy-*n*-butyric acid which has a mercury content of 53.3 per cent. The mercury content of the samples prepared varied from 54 to 59 per cent. This behavior might have been due to the presence of a mixture of the two substances or it might have been occasioned by the formation of a polymeric intermolecular salt. In the case of cinnamic acid we have evidence supporting the latter view. In this case the instability and insolubility of the crotonic acid addition product make it almost impossible to obtain experimental evidence supporting either of the above explanations. The matter therefore was not pursued further. The position of attachment of the mercury and the methoxyl group was shown by reducing the addition product with hydrogen sulfide. An 80 per cent yield of β -methoxy-*n*-butyric acid was obtained.

The conversion of the crotonic acid-mercuric acetate addition product into bromomethoxybutyric acid was first attempted by

brominating a chloroform suspension of the compound. The results were unsatisfactory since a poor yield of the desired product was obtained together with a considerable amount of a highly lachrymatory liquid. The bromination of α -bromomercuri- β -methoxy-*n*-butyric acid was the second reaction which we desired to try. The addition product was readily soluble in aqueous potassium bromide, yielding a solution of potassium α -bromomercuri- β -methoxy-*n*-butyrate. Acidification of this solution, even with dilute acetic acid, caused decomposition. The free acid, therefore, could not be prepared. The problem was finally solved very successfully when it was found that the potassium salt in aqueous solution was rapidly brominated by the addition of a solution of bromine in aqueous potassium bromide. A mixture of the α -bromo- β -methoxy-*n*-butyric acids was obtained as the main product. In this way large amounts of the bromo acids can be prepared rapidly and conveniently in excellent yields. The mixture contains 30 to 40 per cent of the precursor of *dl*-threonine.

Several variations in the method of bromination were tried with no appreciable alteration in the nature of the mixture produced.

The mercuration method of preparing aminohydroxy acids is greatly improved by using as the starting material the unsaturated acid instead of its ester, since the procedure is greatly simplified in several places, and especially because the step involving the hydrolysis of a bromo ester is eliminated. That this step is likely to cause trouble is indicated by the failure of Abderhalden and Heyns (3) to prepare α -amino- β -hydroxy-*n*-valeric acid from the corresponding unsaturated ester, owing to the large loss occurring during the hydrolysis of the intermediate ethyl- α -bromo- β -methoxy-*n*-valerate.

At the present time we are studying the limitations of this method by applying the reactions to other unsaturated acids of various types. The mercuration of dibasic unsaturated acids is also being investigated in the hope of finding a convenient method of preparing hydroxyglutamic acid from glutaconic acid.

EXPERIMENTAL

Mercuration of Crotonic Acid—640 gm. (2 moles) of mercuric acetate were partially dissolved in 3 liters of hot methyl alcohol

and treated with 172 gm. (2 moles) of crotonic acid. The flask was shaken vigorously until the mercuric acetate had completely dissolved. On standing at room temperature a crystalline solid began to separate after 2 to 4 hours, and the major part of the addition product had precipitated at the end of 48 hours. The precipitate was filtered and washed with methyl alcohol. The combined washings and filtrate deposited small additional amounts of the product on further standing. The yield (640 gm.) was almost quantitative since only a small amount of salts of mercury was left in solution. The yield cannot be calculated from the weight of the product since its structure is not definitely known. The substance was insoluble in the common organic solvents. It did not melt sharply but appeared to decompose on heating with the formation of a solid, which on further heating decomposed at 170–180°. The initial decomposition temperature varied from 75–125°, increasing with the length of time of the mercuration. The mercury content of the substance was variable but always lower than that required for anhydro- α -hydroxymercuri- β -methoxy-*n*-butyric acid.

*α -Bromo- β -Methoxy-*n*-Butyric Acids*—320 gm. of the addition product were dissolved in a solution of 180 gm. of potassium bromide in 1 liter of water. A small amount of a black precipitate was removed by filtration. The filtrate was placed in a 4 liter beaker, cooled in an ice bath, and exposed to direct sunlight. A solution of 160 gm. of bromine and 180 gm. of potassium bromide in 300 cc. of water was added from a separatory funnel as it was utilized. 10 to 15 minutes were required for the addition of the bromine. No mercuric bromide precipitated, owing to the excess potassium bromide present. After the bromine was added the solution was extracted once with ether to remove a small amount of a lachrymatory substance. 200 cc. of 40 per cent hydrobromic acid were then added, and the solution was extracted six times with 500 cc. portions of ether. A small amount of the bromo acid remained in the aqueous layer and was obtained by concentrating the solution and again extracting it with ether. It is hardly worth while to do so, however.

The ether extracts were combined, washed once with a small volume of water, and dried. The ether was removed, leaving the crude bromo acid. The yield was 175 to 185 gm. (88 to 93

per cent). This crude material was satisfactory for use in the next step. The pure bromo acid can be obtained by fractionation of the crude material under reduced pressure.

When a large quantity of the bromo acid was being prepared, it was more convenient to isolate it in a different manner. After the bromine was added the reaction mixture was treated with hydrogen sulfide, thereby converting the mercuric bromide to mercuric sulfide. The hydrobromic acid formed acidified the solution. The mercuric sulfide was removed by filtration, and the filtrate was concentrated to about one-half its volume and extracted four times with ether. The ether was removed, leaving the crude bromo acid mixture. By this procedure the mercury and the potassium bromide may be recovered.

The brominations may be carried out in the absence of sunlight. However, the time required for 1 mole is 2 to 3 hours.

Samples of the crude bromo acid were kept in a vacuum desiccator for several weeks. Other samples were purified by distillation and then kept in a vacuum desiccator. No crystalline material was obtained.

*α -Amino- β -Methoxy-*n*-Butyric Acids*—The crude bromo acid mixture was aminated in the usual way. The viscous gum obtained on removing ammonia from the reaction mixture was converted into a light brown granular solid by standing under acetone for 1 to 2 days. This product was used without further treatment in preparing the aminohydroxybutyric acids. The pure aminomethoxybutyric acids were obtained by dissolving the solid in the minimum amount of hot water and adding 9 volumes of absolute alcohol. Usually a second such recrystallization yielded an ammonium bromide-free mixture of the amino acids. Considerable loss attends the purification of the aminomethoxybutyric acids, since they are very soluble in water and appreciably soluble in 95 per cent alcohol. It is better, therefore, to convert the crude amination product directly into the mixture of aminohydroxybutyric acids, since the latter are much less soluble in water and in aqueous alcohol, and can be purified without much loss of material.

*α -Amino- β -Hydroxy-*n*-Butyric Acids*—The acetone-extracted amination mixture was refluxed for 2 hours with 6 times its weight of 48 per cent hydrobromic acid. The solution was con-

centrated *in vacuo*. A considerable amount of solid separated during the concentration. This was filtered when the solution had been reduced to about one-third of its original volume. The precipitate consisted almost entirely of ammonium bromide. The filtrate was concentrated further *in vacuo*. Water was added and the solution was concentrated to dryness under reduced pressure. Water was again added and the solution reconcentrated to dryness. This removed most of the hydrobromic acid. The residue was dissolved in water, and treated with ammonium hydroxide until the ammoniacal odor persisted after vigorous shaking. The solution was concentrated until crystals began to appear, and was then warmed on the steam cone. 4 volumes of alcohol were added. The solution was allowed to stand overnight at room temperature, and the precipitated amino acids and ammonium bromide were removed by filtration. Recrystallization of the mixture from 80 per cent alcohol yielded the pure aminohydroxybutyric acids. Small additional amounts of the amino acids were obtained by concentrating the filtrates and repeating the process. The over-all yields, calculated on the crotonic acid used in the first step, ranged from 45 to 55 per cent.

SUMMARY

A simple, rapid method is described for the conversion of crotonic acid into a mixture of the α -bromo- β -methoxy-*n*-butyric acids. This material contains 30 to 40 per cent of the precursor of *dl*-threonine and is used in preparing the corresponding mixtures of the aminomethoxybutyric acids and of the aminohydroxybutyric acids. Methods of separating these will be described in the succeeding paper.

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SYNTHESIS OF α -AMINO- β -HYDROXY-*n*-BUTYRIC ACIDS

IV. SEPARATION OF MIXTURES OF THE TWO FORMS AND PREPARATION OF *d*(-)- AND *l*(+)-THREONINE*

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In the preceding paper (2) we described a simple method of preparing a mixture of *dl*-threonine and *dl*-allothreonine¹ and a mixture of *dl*-O-methylthreonine and *dl*-O-methylallothreonine. Since all attempts to prepare pure *dl*-threonine directly had failed (2, 3), we decided to investigate the possibility of separating these mixtures. In the present paper we describe a satisfactory method of preparing pure *dl*-threonine based on the separation of a mixture of *dl*-O-methylthreonine and *dl*-O-methylallothreonine. We also report the synthesis of *d*(-)- and *l*(+)-threonine.

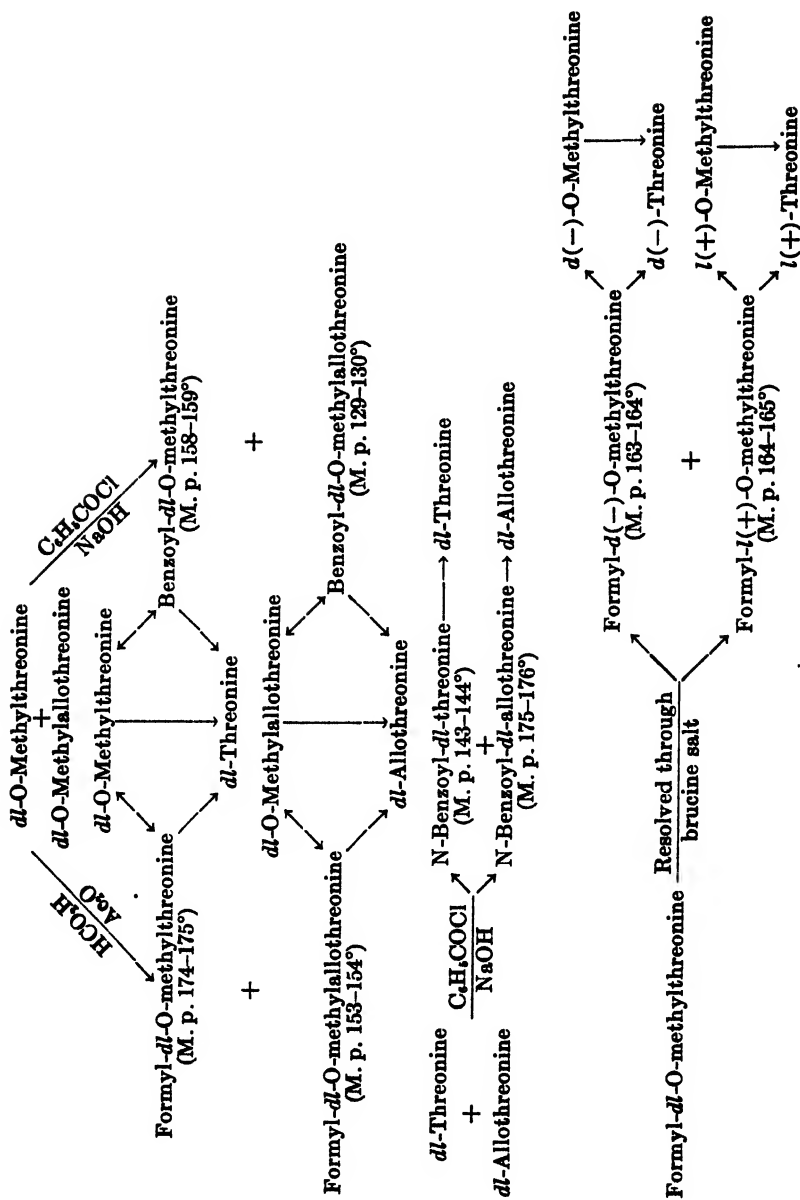
Since previous experiments had shown that the amino acids in question cannot be separated by recrystallization, the present work was carried out on formyl and benzoyl derivatives. The benzoylation of a mixture of *dl*-threonine and *dl*-allothreonine was first studied. Unfortunately the less soluble derivative was benzoyl-*dl*-allothreonine. The more soluble benzoyl-*dl*-threonine was isolated from the residues only with difficulty. Therefore the separation of the benzoylamino-hydroxybutyric acids does not afford a feasible method of preparing *dl*-threonine.

The formylation and benzoylation of a mixture of *dl*-O-methyl-

* The naturally occurring α -amino- β -hydroxy-*n*-butyric acid has been named *d*(-)-threonine. Its enantiomorph is *l*(+)-threonine (Meyer and Rose (1)).

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¹ The term *dl*-allothreonine is used to designate the *dl*- α -amino- β -hydroxy-*n*-butyric acid which does not contain the natural form. This name expresses the most important biochemical property of the substance—its relationship to *dl*-threonine—and conforms with the nomenclature used in the case of the isoleucines.



threonine and *dl*-O-methylallothreonine were studied next. In this case *dl*-O-methylthreonine gave the less soluble derivatives, so that both formyl- and benzoyl-*dl*-O-methylthreonine were isolated from the reaction products in good yields. Furthermore both of these derivatives were smoothly converted into *dl*-threonine by refluxing with 48 per cent hydrobromic acid. Therefore a convenient method is now available for the synthesis of pure *dl*-threonine.

Formyl-*dl*-O-methylthreonine was resolved by means of brucine and the optically active formyl derivatives were converted into *d*(-)- and *l*(+)-threonine with 48 per cent hydrobromic acid.

The reactions which were carried out are shown in the accompanying diagram. All the compounds listed were isolated from the reaction mixtures in a pure state.

EXPERIMENTAL

The physical constants, analyses, and approximate solubilities of the substances prepared are shown in Tables I and II.

Formyl-dl-O-Methylthreonine—A crude mixture of the aminomethoxybutyric acids was prepared as previously described (2). This material was extracted twice with 5 volumes of absolute alcohol, which removed colored impurities and most of the ammonium bromide. The partially purified mixture was then formylated by the method of Fruton and Clarke (4). The resulting solution was concentrated to dryness and the residue was recrystallized from the minimum volume of hot water. The precipitate was crude formyl-*dl*-O-methylthreonine. A second recrystallization from hot water gave a pure product. By working up the mother liquors a yield of 14 to 18 gm. of the derivative was obtained per mole of crotonic acid used in the first step.

*Formyl-dl-O-Methylallothreonine*²—The filtrates from formyl-

² In the first feeding trials carried out on a synthetic aminohydroxybutyric acid (5) the substance used was prepared from recrystallized bromomethoxybutyric acid and was found to possess no growth-promoting activity. Therefore we aminated a crude bromomethoxybutyric acid sample, formylated the amino acid thus obtained, and treated the unpurified formyl derivative with acetic anhydride and sodium hydroxide (du Vigneaud and Meyer (6) had shown that formyl-*l*-phenylalanine was racemized by this treatment). The product on refluxing with 48 per cent hydro-

dl-O-methylthreonine were concentrated to dryness and the residue was extracted with boiling acetone. The acetone solution was concentrated and two or three crystalline fractions were removed. Any fraction which melted at 135–140° or above yielded pure formyl-*dl*-O-methylallothreonine on recrystallization from absolute alcohol.

If any considerable quantity of formyl-*dl*-O-methylallothreonine is desired, it is more readily prepared by formylating pure *dl*-O-methylallothreonine than by working up the mother liquors from formyl-*dl*-O-methylthreonine.

Benzoyl-dl-O-Methylthreonine—A crude mixture of the aminomethoxybutyric acids and ammonium bromide was prepared as previously described (2). The material obtained from 1 mole of crotonic acid was placed in a 5 liter flask and made alkaline with 2 N sodium hydroxide. The ammonia was removed by concentration *in vacuo*. Sufficient 2 N sodium hydroxide was added to make an approximately 0.3 N solution. This was benzoylated in the usual manner with 280 gm. of benzoyl chloride and 2300 cc. of 2 N sodium hydroxide. After the benzoylation was completed a slight excess of concentrated hydrochloric acid was added. The precipitate was removed by filtration, washed with a small volume of cold water, and dried. The filtrate and washings contained mainly benzoyl-*dl*-O-methylallothreonine which was isolated as described later. The precipitate was extracted twice with 2 liter portions of hot, high boiling petroleum ether. The oil remaining solidified on cooling. The solid was dried on a suction filter and recrystallized from the minimum amount of benzene. The filtrate contained some benzoyl-*dl*-O-methylallothreonine and was worked up as described in the next section. The precipitate was extracted

bromic acid yielded a mixture of aminohydroxybutyric acids which supported the growth of rats when included in the diets to the extent of 2.5 to 4 per cent. Since we were not then aware that the crude bromomethoxybutyric acid was a mixture of the two forms, the assumption was made that the acetic anhydride-sodium hydroxide treatment had converted formyl-*dl*-O-methylallothreonine partially into formyl-*dl*-O-methylthreonine. However, it has since been found that the action of acetic anhydride and sodium hydroxide on pure formyl-*dl*-O-methylallothreonine causes only a slight amount of rearrangement. Therefore it appears possible that the crude formyl derivative used in the original experiments was a mixture of the two forms.

TABLE I
Analyses and Constants

	M. p.	[α] _D ²⁰ degrees	Formula	N analyses		Neutral equivalent	
				Calculated	Found	Calculated	Found
				per cent	per cent		
Formyl- <i>dl</i> -O-methylallothreonine.....	153-154		C ₈ H ₁₁ O ₄ N	8.70	8.61	161	161
Formyl- <i>dl</i> -O-methylthreonine.....	174-175		"	8.70	8.74	161	162
Benzoyl- <i>dl</i> -O-methylallothreonine.....	129-130		C ₁₂ H ₁₅ O ₄ N	5.91	5.90	237	235
Benzoyl- <i>dl</i> -O-methylthreonine.....	158-159		"	5.91	5.98	237	236
Benzoyl- <i>dl</i> -allothreonine.....	175-176		C ₁₁ H ₁₃ O ₄ N	6.28	6.14	223	222
Benzoyl- <i>dl</i> -threonine.....	143-144		"	6.28	6.15	223	221
<i>dl</i> -O-Methylallothreonine.....	230-233		C ₈ H ₁₁ O ₃ N	10.53	10.59		
<i>dl</i> -O-Methylthreonine.....	215-218		"	10.53	10.62		
<i>dl</i> -Allothreonine.....	237-239		C ₈ H ₁₁ O ₃ N	11.76	11.70		
<i>dl</i> -Threonine.....	227-229		"	11.76	11.64		
Brucine formyl- <i>d</i> (-)-O-methylthreonine.....	186-188	-19.4	C ₉ H ₁₃ O ₄ N ₂	7.57	7.68		
" formyl- <i>l</i> (+)-O-methylthreonine.....	139-141	-21.5	"	7.57	7.80		
Formyl- <i>d</i> (-)-O-methylthreonine.....	163-164	+11.8	C ₈ H ₁₁ O ₄ N	8.70	8.74	161	160
Formyl- <i>l</i> (+)-O-methylthreonine.....	164-165	-11.9	"	8.70	8.54	161	160
<i>d</i> (-)-O-Methylthreonine.....	214-216	-37.8	C ₈ H ₁₁ O ₃ N	10.53	10.60		
<i>l</i> (+)-O-Methylthreonine.....	214-216	+38.2	"	10.53	10.51		
<i>d</i> (-)-Threonine.....	251-253*	-28.3	C ₈ H ₁₁ O ₃ N	11.76	11.70		
<i>l</i> (+)-Threonine.....	251-252*	+28.4	"	11.76	11.64		
Benzoyl- <i>d</i> (-)-threonine.....	147-148	+25.1	C ₁₁ H ₁₃ O ₄ N	6.28	6.40	223	221
Benzoyl- <i>l</i> (+)-threonine.....	147-148	-25.5	"	6.28	6.21	223	222

* The amino acid softened and darkened somewhat at 220-225°.

TABLE II
*Solubilities of Aminomethoxybutyric Acids, Aminohydroxybutyric Acids, and Their Derivatives**

	Water	Ethyl alcohol†	Acetone	Ethyl acetate	Benzene
Formyl- <i>dl</i> -O-methylallothreonine.....	(114.0) 13.28	(58.0) 9.16	(5 0) 1.66	(1.6) 0.28	(0.1) 0.002
Formyl- <i>dl</i> -O-methylthreonine.....	(32.0) 3.89	(26.0) 3.63	(2.0) 0.67	(1.0) 0.17	(0.05) 0.012
Benzoyl- <i>dl</i> -O-methylallothreonine.....	(8.5) 0.84	(100 0) 20.86	(43.0) 11.20	(32.0) 4.19	(4.4) 0.15
Benzoyl- <i>dl</i> -O-methylthreonine.....	(1.8) 0.22	(50.0) 10.44	(11 0) 4.47	(5.5) 1.14	(0 9) 0.05
Benzoyl- <i>dl</i> -allothreonine.....	(5.0) 0.85	(43 0) 7.28	(3.5) 1.51	(1.2) 0.24	(0.02) 0.001
Benzoyl- <i>dl</i> -threonine.....	(67.0) 2.06	(86 0) 24.60	(15 0) 6.09	(4.5) 1.02	(0 06) 0.003
<i>dl</i> -O-Methylallothreonine.....	(80.0) 40.7	0.42			
<i>dl</i> -O-Methylthreonine.....	(105.0) 41.9	0.75			
<i>dl</i> -Allothreonine.....	(31.0) 13.9	0.03			
<i>dl</i> -Threonine.....	(55.0) 20.1	0.07			

* The solubilities are expressed in terms of gm. of solute per 100 cc. of solution at 25°. The figures in parentheses are the approximate solubilities at 80° or at the boiling point of the solvent if the solvent boils below 80°.

† 85 per cent.

once with hot petroleum ether. The residue was practically pure benzoyl-*dl*-O-methylthreonine. It crystallized readily from hot water in shiny needles. The yield was 26 to 30 gm. and a small additional amount (3 to 4 gm.) was obtained in working up the filtrates as described in the next section.

Benzoyl-dl-O-Methylallothreonine—Pure *dl*-O-methylallothreonine (prepared from recrystallized bromomethoxybutyric acid) was benzoylated with benzoyl chloride and sodium hydroxide in the usual manner. The filtrate from the benzoic acid was cooled in an ice box overnight. The crystalline solid was removed by filtration and combined with the small residue which remained after extracting the original precipitate with hot, high boiling petroleum ether. Two recrystallizations from benzene gave the pure benzoyl derivative. The yield was 75 per cent of the theoretical amount. Benzoyl-*dl*-O-methylallothreonine crystallizes very readily in beautiful needles from a hot benzene solution.

Benzoyl-*dl*-O-methylallothreonine was also obtained from the filtrates described in the preceding section. The aqueous filtrate was cooled overnight in an ice box and the precipitate was removed by filtration. The filtrate was concentrated until a solid began to separate, and was then cooled in an ice box, yielding a second crop of crystals. The benzene filtrate was concentrated to dryness and the three solid fractions were combined and extracted once with hot, high boiling petroleum ether. The residue was extracted with 4 volumes of hot acetone. The undissolved material, consisting mainly of sodium chloride, was removed by filtration and the acetone solution was cooled in an ice box overnight. The precipitate was crude benzoyl-*dl*-O-methylthreonine. The filtrate was concentrated to dryness and the residue was dried and recrystallized from 30 volumes of hot benzene. Several recrystallizations were required to obtain pure benzoyl-*dl*-O-methylallothreonine.

N-Benzoyl-dl-Allothreonine—The mixture of *dl*-threonine and *dl*-allothreonine previously described (2) was benzoylated by the method of Sørensen and Andersen (7) with 3 moles of benzoyl chloride per mole of amino acid. The benzoyl derivatives are sufficiently soluble in water to prevent their precipitation with the benzoic acid. Therefore it is not necessary to work up the benzoic acid fraction. The filtrate and washings from the benzoic

acid were concentrated to about one-half of the original volume. The solution was then placed in an ice box for 24 hours. The precipitate was removed by filtration, dried, and extracted with hot, high boiling petroleum ether. The residue was crude N-benzoyl-*dl*-allothreonine. It was purified by recrystallization from hot water. The pure compound melted at the temperature reported by Abderhalden and Heyns (8). 110 to 120 gm. of pure N-benzoyl-*dl*-allothreonine were obtained from 1 mole of the mixture of aminohydroxybutyric acids.

N-Benzoyl-dl-Threonine—6 gm. of pure *dl*-threonine (prepared from formyl-*dl*-O-methylthreonine) were benzoylated by the method of Sorensen and Andersen (7). The filtrate from the benzoic acid was concentrated to one-third of the original volume. The solution was placed in an ice box overnight. The precipitate was removed by filtration and dried. Benzoic acid was removed with petroleum ether and the residue was extracted with 30 volumes of boiling ethyl acetate. The solution was filtered and the filtrate was cooled in an ice box overnight. Practically pure N-benzoyl-*dl*-threonine precipitated. It was purified by recrystallization from ethyl acetate. The yield was 9.5 gm. (85 per cent of the theoretical amount).

N-Benzoyl-*dl*-threonine was also isolated from the filtrates from N-benzoyl-*dl*-allothreonine. These were concentrated to a small volume, causing an oil to separate. After standing for some time or seeding with N-benzoyl-*dl*-threonine the material crystallized. It was removed by filtration, dried, extracted with hot, high boiling petroleum ether, and recrystallized several times from ethyl acetate. 15 to 20 gm. of pure N-benzoyl-*dl*-threonine were obtained from 1 mole of the mixture of aminohydroxybutyric acids.

Preparation of dl-O-Methylthreonine and dl-O-Methylallothreonine

From Formyl Derivatives—The formyl derivative was refluxed 3 hours with 10 volumes of 1 N hydrobromic acid. The solution was concentrated *in vacuo* to a small volume. Water was added and the solution was concentrated to dryness. The residue was dissolved in 30 cc. of absolute alcohol and concentrated ammonium hydroxide was added until a faint odor of ammonia persisted after vigorous shaking. On cooling overnight the amino acid precipi-

tated. It was purified by recrystallization from 95 per cent alcohol.

From Benzoyl Derivatives—The benzoyl derivative was refluxed 12 hours with 20 volumes of 10 per cent hydrochloric acid. The benzoic acid was extracted with ether and the aqueous solution was concentrated to dryness *in vacuo*. Water was added and the concentration was repeated. The residue was worked up in the manner described for the formyl derivative. The benzoyl derivatives were not readily hydrolyzed and a small amount was usually left unattacked by this treatment. It is unadvisable, however, to use more strenuous conditions, owing to the danger of splitting the ether linkage also.

Preparation of dl-Threonine and dl-Allothreonine

From Formyl-dl-O-Methylthreonine and Formyl-dl-O-Methylallothreonine—The formyl derivative was refluxed 2 hours with 10 volumes of 48 per cent hydrobromic acid. The hydrobromic acid was removed and the amino acid was isolated in the usual way. The yields on this step ranged from 80 to 90 per cent of the theoretical amount.

From Benzoyl-dl-O-Methylthreonine and Benzoyl-dl-O-Methylallothreonine—The benzoyl derivative was refluxed 2.5 hours with 15 volumes of 48 per cent hydrobromic acid. The solution was cooled, diluted with an equal volume of water, and extracted twice with ether to remove benzoic acid. The amino acid was then isolated in the usual way. The yields on this step ranged from 80 to 90 per cent of the theoretical amount.

From N-Benzoyl-dl-Threonine and N-Benzoyl-dl-Allothreonine—The derivative was refluxed 4 hours with 15 volumes of 20 per cent hydrobromic acid. The benzoic acid was extracted with ether and the amino acid was isolated in the usual way. The yield was 85 per cent of the theoretical amount.

Resolution of Formyl-dl-O-Methylthreonine—63 gm. of the formyl derivative, dissolved in 665 cc. of absolute alcohol, were added to a solution of 154 gm. of anhydrous brucine in 1570 cc. of hot absolute alcohol. The solution was left in an ice box for 48 hours. The less soluble brucine salt crystallized and was removed by filtration. It was recrystallized from 10 volumes of boiling absolute alcohol. The yield was 96.9 gm. (89 per cent of the theoretical amount).

On concentrating the mother liquor, the more soluble brucine salt was obtained. The crude material melted at 175–182°. It was purified by three recrystallizations from 5 volumes of boiling absolute alcohol. The yield was 73 gm. (67 per cent of the theoretical amount).

Formyl-l(+)-O-Methylthreonine—74.3 gm. of the less soluble brucine salt were dissolved in 1 liter of water and treated with a slight excess of 1 N sodium hydroxide. The brucine was extracted with three 500 cc. portions of chloroform and the alkali was neutralized with an equivalent amount of sulfuric acid. The solution was concentrated *in vacuo*, until crystals of the formyl derivative began to appear. After cooling in an ice box overnight the crystals were removed by filtration and dried. The yield was 19 gm. The filtrate was concentrated and cooled again, yielding 2 gm. more of the formyl derivative.

Formyl-d(-)-O-Methylthreonine—67 gm. of the more soluble brucine salt were decomposed in the manner described for the less soluble salt. By carefully working up the mother liquor 17 gm. (83 per cent of the theoretical amount) of formyl-d(-)-O-methylthreonine were obtained.

When 0.5 gm. each of the two optically active formyl derivatives was mixed and crystallized from water, the crystals obtained were inactive and melted at 171–173°. The mother liquor was also optically inactive.

d(-)- and l(+)-O-Methylthreonine—The formyl derivatives were hydrolyzed and the reaction products were worked up in the usual way. Considerable difficulty was encountered in crystallizing the amino acids, since the addition of absolute alcohol to warm aqueous solutions of the substance usually caused a thick gel to form. They were finally obtained in a crystalline form by slow evaporation of aqueous alcohol solutions.

d(-)- and l(+)-Threonine—Formyl-d(-)- and formyl-l(+)-O-methylthreonine were converted directly into d(-)- and l(+)-threonine in the usual manner. No difficulty was encountered in crystallizing the amino acids from 80 per cent alcohol. The yield on this step ranged from 80 to 90 per cent of the theoretical amount.

N-Benzoyl-d(-)- and N-benzoyl-l(+)-threonine were prepared by the method of Sørensen and Andersen (7). The derivatives

were present in the filtrates obtained by acidifying the benzoylation mixture and removing the benzoic acid by filtration. The filtrates were concentrated and cooled in an ice box. The precipitates were removed by filtration, dried, extracted with hot petroleum ether, and recrystallized from ethyl acetate. Both of the benzoyl derivatives melted at 147–148°. McCoy, Meyer, and Rose (9) reported a melting point of 151° for the N-benzoyl derivative of natural threonine.

SUMMARY

1. Formyl- and benzoyl-*dl*-O-methylthreonine are readily isolated as the less soluble products respectively of the formylation and benzoylation of a mixture of the aminomethoxybutyric acids. *dl*-Threonine is prepared in a good yield by refluxing either of the above substances with 48 per cent hydrobromic acid.

2. Benzoyl-*dl*-threonine is isolated in a poor yield as the more soluble product of the benzoylation of a mixture of the amino-hydroxybutyric acids.

3. Formyl-*dl*-O-methylthreonine is readily resolved by means of brucine. The optically active formyl derivatives are smoothly converted into *d*(-)- and *l*(+)-threonine with 48 per cent hydrobromic acid.

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PIMELIC ACID AS A GROWTH ACCESSORY FOR THE DIPHTHERIA BACILLUS

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In the course of a series of studies on nutritional requirements of certain pathogenic bacteria (1), it was found that the ether extract of an acidified tissue extract (liver) possessed a marked growth-stimulating effect for a strain of the diphtheria bacillus. It was possible, through the courtesy of Dr. Y. Subbarow of the Department of Biological Chemistry at Harvard, and of the Lederle Laboratories, Inc., Pearl River, New York, to obtain sufficient material to demonstrate that the substance concerned was probably an organic acid, and that it could be distilled *in vacuo*, the distilled esters still showing the effect on growth after saponification.

The amount of the acid material necessary to induce full growth on suitable control media was so small as to suggest that the substance was in the nature of a growth accessory. The yield of distilled esters from a concentrate representing 60 kilos of liver tissue was about 0.25 gm., and obviously consisted of several different substances. Consequently there appeared to be little chance of successfully isolating the substance unless a very large amount of liver extract could be worked up. It is possible that this could have been arranged, but in the meantime it was found that urine furnished an even better source of the active substance than liver—particularly the urine of the horse and the cow. The same fact had been discovered to be true of another acid substance involved in bacterial nutrition, the *sporogenes* vitamin of Knight and Fildes (2), by Pappenheimer (3). This latter substance, however, which it was possible to test, through the courtesy of Dr. Pappenheimer, proved to be without effect on the diphtheria bacillus.

Isolation of the active material from urine was therefore undertaken. Preliminary tests served to indicate the general method to be followed, and that a quantity of 100 gallons of horse or cow urine should be sufficient to yield an amount of distilled esters adequate for separation. It appeared that the laborious extraction of this quantity of urine might be avoided if suitable by-products could be obtained from the preparation of theelin, but although several industrial companies and research workers engaged in the production of this material very kindly supplied concentrates for testing, none showed any considerable activity. This was explained by the finding that the active acid was extracted from urine by butyl alcohol or ether, but not by solvents of the type of chloroform, benzene, or petroleum ether, and, further, that when butyl alcohol was used, the acid was esterified upon concentration, even *in vacuo*, and could not be removed from the solvent with cold dilute alkali.

The extraction and concentration of 100 gallons of cow urine were therefore carried through as described below. No difficulties, other than mechanical, were encountered in the application of the methods worked out on a small scale, and although losses occurred at almost every step in the procedure, as checked by growth tests, a yield of 120 gm. of dry methyl esters, giving 83 gm. of distillate, was obtained.

This distillate showed itself to be an extremely complex mixture from which there was little hope of separating the active substance by the usual type of fractional distillation in a sufficiently pure form to identify it. In general, the low boiling esters were more active than the higher, and it was found that the material was sufficiently stable to withstand distillation at atmospheric pressure, the esters coming over at about 250° being somewhat more active than the lower and higher fractions, but without more knowledge of the nature of the substance repeated redistillation under these conditions seemed unwise.

At this stage, the writer was fortunate in obtaining the cooperation of Dr. Max Rittenberg of the Department of Biological Chemistry, College of Physicians and Surgeons, New York. By means of an extremely effective vacuum distilling column, the details of the construction and operation of which Dr. Rittenberg will publish shortly, he was able to separate about 3 gm. of an

Already highly active fraction of the esters into ten fractions, one of which later proved to be nearly pure dimethyl pimelate.

With an apparatus made according to Dr. Rittenberg's specifications, the writer then worked over the whole preparation in small portions, gradually concentrating the activity into a smaller bulk, until finally about 2.5 cc. of very highly active ester were obtained which solidified to a mass of white crystals in dry ice, melting again at room temperature. Half of this fraction, upon saponification and after several recrystallizations, gave 0.30 gm. of crystals melting at 104° , which were identified as pimelic acid. They were highly active in the growth test with the diphtheria bacillus; details of this phase of the subject will be published elsewhere (4). Commercial pimelic acid (Eastman) showed the same biological activity. It increases growth of the culture to maximum in an amount of 0.02 to 0.04 microgram per cc. of medium.

Although the remainder of the ester material has not been systematically examined, one other acid, azelaic, has been isolated from it, in the course of this work. Neither this acid, nor any of the other dibasic acids of lower molecular weight, shows the growth-stimulating action of pimelic acid. Dibasic acids of higher molecular weight than azelaic acid have not been available for testing.

So far as can be learned, neither pimelic nor azelaic acid is recognized as a normal constituent of urine or of animal tissues. As to the identity of the diphtheria bacillus growth-stimulating substance in liver with pimelic acid, it can now merely be stated that through the stage of distillation of the methyl esters, including stability to distillation at atmospheric pressure and approximate range of boiling point, the behavior has been the same. In experiments now in progress, other quite unrelated bacterial growth factors of liver are being studied, and if, in the course of this work, sufficient ether-soluble material becomes available, an attempt will be made to isolate and identify the pimelic acid.

EXPERIMENTAL

Fresh cow urine, in 10 gallon lots, was acidified by the careful addition of 1000 cc. of concentrated H_2SO_4 . A few cc. of caprylic alcohol helped control the foaming. It was allowed to stand over-

night, resulting in the separation of a considerable amount of hippuric acid. After stirring in an ounce or two of kieselguhr, the material was then passed through pleated filters into three 5 gallon bottles. The latter were then nearly filled with commercial butyl alcohol and mixed for about 15 minutes with a current of air. Separation of the alcohol took place quickly, and the aqueous layers were siphoned off into empty 5 gallon bottles. Two further extractions, three in all, with butyl alcohol were carried on in the same way, and the aqueous residue was then discarded.

The butyl alcohol extracts were combined, also in 5 gallon bottles, and a suspension of NaHCO_3 in water was added carefully to avoid loss by foaming, until after thorough mixing a definitely alkaline reaction to litmus persisted. The solution of sodium salts was then siphoned off and saved until the entire 100 gallons had been extracted. This now amounted to 10 gallons. It was a nearly black, moderately alkaline solution.

The combined NaHCO_3 extracts were concentrated *in vacuo* to about half, in order to remove as much butyl alcohol as possible. Acidification with HCl to Congo red paper, requiring about 3000 cc. of concentrated acid, caused the separation of a large quantity of hippuric acid. After standing overnight this was filtered off on a large Buchner funnel, pressed dry, suspended in about 8 liters of water, and refiltered.

The solution of acids was now again extracted with butyl alcohol, making six extractions with 2 liters the first time and 1 liter in subsequent treatments.

It was next desired to carry out a $\text{Ba}(\text{OH})_2$ -alcohol precipitation on the material which had been found to remove much pigment and extraneous material, but it proved impossible to extract the butyl alcohol solution with $\text{Ba}(\text{OH})_2$ solution because of the formation of a heavy precipitate and an emulsion. The matter was therefore approached indirectly. The solution was extracted with sufficient NH_4OH solution to render it alkaline, and then washed out three times with water containing a small amount of ammonia. The combined ammoniacal extracts were concentrated *in vacuo* to remove excess NH_3 and butyl alcohol, and an excess of crystalline baryta was then added, and distillation continued until the remainder of the NH_3 had been removed. The

solution of Ba salts, containing considerable precipitate, was diluted to 5000 cc., 2 volumes of 96 per cent ethyl alcohol were added, and the solution was allowed to stand overnight.

The very bulky, yellow clay-like precipitate was removed on a large Buchner funnel, resuspended, and partially dissolved in 4000 cc. of water and again separated by 2 volumes of alcohol, followed by filtration. The filtrates were combined, and the alcohol removed by vacuum distillation. The aqueous solution of Ba salts was acidified with HCl and extracted six times with butyl alcohol; these extracts in turn were extracted once with strong, and five times with weak NaOH solution.

The quantities had been so arranged that the aqueous solution of sodium salts now occupied a volume of about 2 liters. In order to remove most of the butyl alcohol, it was extracted four times with relatively small amounts of ether—while still alkaline. It was then acidified with HCl and extracted repeatedly with peroxide-free ether.

The ether extract was dried with Na_2SO_4 , and the solvent removed, finally *in vacuo*, leaving 270 gm. of syrupy acids.

To this were added 3000 cc. of absolute methyl alcohol and dry HCl gas was run in until an increase in weight of 90 gm. resulted. The mixture was allowed to stand overnight at room temperature and then refluxed for half an hour on the water bath. The alcohol and excess HCl were removed *in vacuo*, leaving 370 gm. of a thick syrup.

After cooling, about 5000 cc. of ether were added to the syrup, only a part of which dissolved. After thorough shaking in a separatory funnel, the insoluble oil was removed. (This probably consisted largely of the ester hydrochlorides of certain nitrogen-containing acids. From it, for example, a considerable amount of phenaceturic acid was later obtained.)

The ethereal solution was washed quickly with NaHCO_3 solution, 0.1 N NaOH solution, and very dilute $(\text{NH}_4)_2\text{SO}_4$, and finally dried over Na_2SO_4 . After removal of the ether, the residual esters weighed 120 gm.

This material was distilled in an oil pump vacuum of less than 1 mm. pressure from a Claisen flask fitted to a Fischer triangle; a dry ice trap was used. The fractions listed in Table I were obtained.

Growth tests showed greatest activity in Fractions I and II and further separation has been carried out only on these, the other three remaining so far uninvestigated.

Fractions I and II were fractionally distilled under atmospheric pressure. The esters of Fraction I, distilled first, were separated into six further fractions and a residue; Fraction II, added to the residue, was then distilled, fractions of the same boiling point

TABLE I
Oil Pump Distillation of Methyl Esters

Fraction No.	Temperature of vapors	Temperature of bath	Weight
	°C.	°C.	gm.
I	81- 84	To 114	15
II	93-109	" 135	19
III	110-130	" 155	25
IV	130-149	" 175	11
V	158-163	" 195	13

TABLE II
Water Pump Redistillation of Lower Boiling Esters

Fraction	Temperature of vapors	From Fraction I	From Fraction II
	°C.	gm.	gm.
A	198-210	2.5	
B	210-220	5.9	
C	220-225	2.2	
D	228	0.8	
E	233-245	1.4	3.0
F	245-255	0.5	5.0
G	255-265		6.3
H	265-(bath to 300)		4.5
Total. Fractions I and II.....		32.1	

being mixed with those of Fraction I. The separation was as given in Table II. All fractions were active, but Fractions E, F, and G were the best. The residue of 3 to 4 cc. had little activity.

3.1 gm. of Fraction F were then distilled by Dr. Rittenberg; the fractions in Table III resulted. These fractions, except the residue, which was brown, were colorless oils. The first four showed no evidence of crystallizing when cooled in dry ice and

alcohol, whereas the remainder solidified to white crystalline masses. The first three had a strong and characteristic aromatic odor, somewhat resembling methyl benzoate. The remainder have very faint, sweetish odors, suggestive of malonic ester. Of these ten fractions, F-4, F-5, and F-6 had the highest activity.

Fractions A to G were then distilled in an apparatus similar to Dr. Rittenberg's. The details of the distillations need not be given, but it was attempted to concentrate the material corresponding to his Fraction F-5 (the first to solidify in dry ice). After passing all of the material through the apparatus once, the various fractions corresponding to his Fractions F-4, F-5, and F-6

TABLE III

Careful Refractionation of Active Ester Fraction At 0.04 Mm. of Hg Pressure

Distillation was carried on at about 0.04 mm. pressure.

Fraction No.	Temperature of vapors	Approximate quantity
	°C.	cc.
F-1	? (Low)	0.7
F-2	? "	0.3
F-3	50-74	0.4
F-4	72	0.2
F-5	78-80	0.3
F-6	82	0.3
F-7	80	0.15
F-8	86	0.2
F-9	87	0.1
F-10 (Residue)		

were combined, and again distilled. In this way were finally obtained 2.5 cc. of highly active material, the lowest boiling fraction to solidify in dry ice.

These distillations were carried out with water-pump vacuum at about 7 mm. pressure, since it was felt that the boiling points with an oil pump were unnecessarily low. The fraction in question boiled under these conditions at 110-117°.

Examination of Active Fraction

27.45 mg.: 59.40 mg. CO₂ and 21.50 mg. H₂O = C 59.00%, H 8.76%
 24.20 " : 52.00 " " " 18.70 " " = " 58.60%, " 8.65%
 (CH₂)₄(COOCH₃)₂. Calculated. C 57.40, H 8.57

A portion of the esters weighing 1.13 gm. was dissolved in 40 cc. of ethanol and 10 cc. of water. After adding 2.0 gm. of NaOH, the mixture was refluxed 1 hour. Enough HCl was added to render the solution acid to Congo red, and it was evaporated to dryness *in vacuo*. The free acid was extracted from the NaCl with ether and evaporated to dryness.

The yield of acid, which was liquid at about 60° but solid at room temperature, was 0.93 gm. This was dissolved in about 10 cc. of hot butyl ether, and on cooling a heavy white crust of granular crystals separated. Since a small amount of suspended matter had been present in the butyl ether solution, the crystals were next dissolved in hot chloroform and filtered, and the chloroform evaporated. The residue weighed 0.78 gm. It was then crystallized three times more from butyl ether, giving a final yield of 0.30 gm. of coarse, well formed crystals, which melted sharply at 104°, uncorrected. Nitrogen, sulfur, and halogens were absent.

19.05 mg.: 37.7 mg. CO₂ + 13.0 mg. H₂O = C 53.96%, H 7.64%
 21.00 " : 41.6 " " + 14.3 " " = " 54.02%, " 7.62%
 (CH₂)₆(COOH)₂. Calculated. C 52.46, H 7.56

The poor agreement in carbons, both here and in the case of the esters, led us to believe, at this stage of the work, that we were dealing with a more complex type of substance.

Titration of the acid gave the following: 15.8 mg. = 19.64 cc. of 0.01 N NaOH, giving a titration equivalent of 80.5; calculated for pimelic acid 80.0.

The phenylphenacyl ester of the material was prepared as follows: The 15.8 mg. lot which was titrated with 0.01 N NaOH was used; the solution was evaporated to dryness *in vacuo*, after adding a crystal of the free acid to restore slight acidity. A few tenths of a cc. of H₂O and 1 cc. of alcohol were added, and 60 mg. of phenylphenacyl bromide. The mixture was gently refluxed for 4 hours, small amounts of alcohol being added to keep the separating crystals in solution. After cooling, the product was twice recrystallized from alcohol, in which it was almost insoluble in the cold. Yield, 13.0 mg. M.p. 147° (uncorrected). Molecular weight determinations by the camphor method resulted in this way: free acid, 1.152 mg. with 9.920 mg. of camphor, m.p. 158.2°; phenylphenacyl ester, 1.140 mg. with 10.275 mg. of camphor, m.p. 171.4°, m.p. (camphor) 178.4°.

The molecular freezing point depression of the camphor used

was determined by means of duplicate determinations with acetanilide and azelaic acid. The values found with acetanilide were 325 and 344, with azelaic acid 294 and 302.

With the average of these four figures, 316, the above observations led to the molecular weights of 181 and 510 for the free acid and its phenylphenacyl ester. Calculated for pimelic acid, it was 160.1, and for its diphenylphenacyl ester, 524.

These figures, all in rather poor agreement with the theoretical, indicated that the substance was a dibasic acid with only 4 oxygen atoms, and comparison with the melting points of the known dibasic acids indicated that it was probably pimelic acid. A mixed melting point determination with once recrystallized Eastman's pimelic acid gave these figures: unknown 104.2°, mixture 104.6°, pimelic acid 104.8°.

The identification was confirmed by preparing the phenylphenacyl derivative of synthetic pimelic acid by the procedure given above, and carrying out mixed melting point determinations with the natural derivative: unknown 147.5°, mixture 147.5°, pimelic acid derivative 147.5°.

Effect of the Urine Acid and Pimelic Acid on Growth of Bacillus Diphtheria

The isolation of this acid was followed at each step with growth tests carried out as outlined below. For exact details of procedure, reference may be made to earlier publications (1). The system was so designed as to give as sharp a contrast as possible between growth on control solutions in the absence of the acid, and in its presence. This particular phase of the general study has extended over nearly a year, and involved the preparation of roughly 3000 test lots of media.

Control Medium

	gm.
Casein-HCl hydrolysate	0.100
Cystine	0.001
Glutamic acid hydrochloride	0.050
NaCl	0.050
$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	0.025
KH_2PO_4	0.0035
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.003
	cc.
Lactic acid (as Na salt)	0.10
Non-ether-soluble fraction of liver extract	0.01

This solution mixed in volumes of 10 cc. with the material to be tested is brought to pH 7.4 to 7.6, sterilized by 10 minutes autoclaving at 10 pounds, and inoculated with the test strain of the diphtheria bacillus. After 48 to 60 hours incubation in a slanted position at 34° growth reaches a maximum. The tubes are steamed 10 minutes to kill the organisms, which are then centrifuged and washed, and the relative amounts of growth estimated by the Pregl micro-Kjeldahl method.

TABLE IV

Comparison of Effect of Acid Isolated from Urine with Synthetic Pimelic Acid on Growth of Diphtheria Bacillus

	Pimelic acid	Tube A	Tube B
	microgram per 10 cc.	mg. bacterial N.	mg. bacterial N
Control + natural pimelic acid....	1.0	4.08	4.05
" + " " " " ...	0.5	3.96	4.05
" + " " " "	0.25	3.61	3.51
" + " " " " ...	0.1	2.48	2.28
" + " " " "	0.05	1.78	1.81
" + " " " "	0.025	1.68	1.63
" + " " " "	0.01	1.46	1.44
" + synthetic " "	1.0	4.01	3.93
" + " " " "	0.5	3.85	3.76
" + " " " "	0.25	3.58	3.54
" + " " " "	0.1	2.14	2.24
" + " " " "	0.05	1.94	1.80
" + " " " "	0.025	1.52	1.62
" + " " " "	0.01	1.49	1.24
"		1.68	1.68

Such tubes of the control solution were mixed with amounts of synthetic pimelic acid and the crystals obtained from urine and the series of determinations shown in Table IV were made.

The irregularities in controls and tubes containing the lowest concentrations of the acid seem to be unavoidable in spite of the greatest care to insure uniformity of solutions and cleanliness of glassware, and probably depend upon phenomena of bacterial variation beyond control. On the whole, a rather remarkable degree of uniformity can be attained by this method over a long period of time. It is clear that the synthetic pimelic acid dupli-

cates the effect of the urine preparation. The effect becomes apparent in a concentration of about 0.005 microgram per cc. of medium and reaches a maximum at about 5 times this concentration. Increasing the amount even up to 1 per cent has no further effect, either accelerating or inhibitory.

Isolation of Azelaic Acid

While this substance shows no effect on the growth of our test organism, it may be of certain general interest to note its isolation in the course of this work. It was obtained as the crystalline hydrazide from a small lot of esters which were being examined as a preliminary to the large scale experiment. The substance was provisionally identified by combustion and Dumas nitrogen determinations carried out by Mrs. Millwood of the Converse Laboratory, Harvard University, then converted to the free acid, and its identity confirmed by mixed melting point determination with the commercial acid. It appears not to be recognized as a normal urinary constituent.

SUMMARY

1. Pimelic acid has been isolated from cow urine in a quantity of approximately 0.6 gm. from 100 gallons. Losses in final crystallization, as well as in the earlier stages of the separation, indicate the presence of a considerably larger amount. Calculated from the effect on growth of the diphtheria bacillus of the original urine as compared with the pure acid, an amount of about 1 mg. per 100 cc. is indicated, or approximately 3.5 gm. in 100 gallons.

2. In suitable control media pimelic acid gives recognizable stimulation of growth of the test strain of diphtheria bacillus in a concentration of 0.005 microgram per cc. of medium, and reaches a maximum in 5 times this concentration.

3. The isolation of azelaic acid from cow urine is reported as an incidental observation.

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SOME PROBLEMS IN TWO-STEP OXIDATION TREATED FOR THE CASE OF PHENANTHRENEQUINONE- SULFONATE

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The fact that the theory of the two-step oxidation has been repeatedly applied to biologically important substances justifies a supplement to this theory. Since a suitable example is at hand, two problems will be dealt with—first, a problem concerning the overlapping of the two steps, and secondly, the question whether at such pH at which no step formation can be observed with the present customary methods there is really no step formation at all or whether the difference from other conditions is only quantitative. Our example will be one of the true quinones, among which biologically occurring representatives are hallachrom (1), juglone and lawsone (2), homogentisic acid (3), lapachol and lomatiol (4), and phthiacol (5, 6).

The problem of two-step oxidation, first elaborated on cationic dyes, has now been successfully studied for anionic dyes; for the indigosulfonates (7), naphthoquinonesulfonate (8),¹ the anthraquinonesulfonates (9), and phthiacol (6). In this paper we shall add another case which, for the purpose of such a study, combines all the advantages presented singly by those previously used and in addition presents other features for the further investigation of the problem.

These favorable properties are (1) that the quinonoid form is stable for the present purpose even in the strongest aqueous solution of NaOH; (2) that the separation of the two steps begins at a

¹ In the previous paper (8), the following error should be corrected. All potentials as indicated in the ordinate of Fig. 1, or in the column " E_m " in Table I, should be written more positive by 0.478 volt. This correction is double the potential of the calomel electrode used in the experiments referred to the normal hydrogen electrode. By mistake, the calomel electrode potential was subtracted from, instead of added to the experimentally observed potentials.

convenient pH range, the crossing point of the potentials lying at pH 10; (3) that it makes possible the pursual of the three normal potentials both to the highest and to the lowest accessible pH. The substance is the 3-monosulfonate of phenanthrenequinone.

Both phenanthrene-2- and phenanthrene-3-sulfonic acids were prepared as the potassium salts by the procedure adequately described by Fieser (10). These were oxidized to the two corresponding phenanthrenequinonesulfonates by the use of chromic acid in glacial acetic acid, details of which are given by Werner (11). The phenanthrenequinonesulfonates were recrystallized as potassium salts. The air-dried salts retain a small amount of water. The analyses of the products dried at 110–120° are as follows: calculated for $C_{14}H_7O_5SK$, S 9.82, K 11.98; found for potassium phenanthrenequinone-2-sulfonate, S 9.47, K 11.36; found for potassium phenanthrenequinone-3-sulfonate, S 9.43, K 11.29.

A simple method for preparing the semiquinone consists in making a suspension of phenanthrenequinone in alcohol, about 1 gm. to 20 cc., and adding about 1 cc. of a strong aqueous sodium hydroxide solution. The yellow suspension rapidly turns to a suspension of the green rhombic plates. If the same experiment is carried out with the phenanthrenequinonesulfonates, corresponding products are formed as dark green needles. In this case, the alkaline alcohol acts as the reductant. The reduction stops at the semiquinone level, probably for two reasons; first, the reducing power of this reductant seems to be just sufficient to this end, and in addition, the very slight solubility of the semiquinone is in favor of its accumulation in crystalline form. When the products are filtered off, they are rapidly reoxidized by air to the yellow quinones. A similar effect has been observed by Klinger (12) who found that phenanthrenequinone in ether in the presence of sunlight is reduced to phenanthrenehydroquinone.

The colors of the various stages of oxidation of the sulfonates are:

	pH < 7	pH > 11
Quinone.....	Yellow	Practically colorless
Quinhydrone.....	Dark (brown ?)*	Intensely brown
Hydroquinone.....	Colorless	Yellow

* The semiquinone can be observed in this pH range only in a mixture with an enormous excess of the intensely yellow quinone and can only be said to have a much darker color than the latter.

The technique of potentiometric titration was the usual one; in most cases oxidative titration of the reduced form with ferricyanide was used. The hydroquinone was prepared in the titration vessel by reduction of the quinone with hydrogen and colloidal palladium.

TABLE I
Potassium Phenanthrenequinone-3-Sulfonate

Temperature 30°. E_m represents the mean normal potential; E_1 , E_2 , normal potential of the lower and of the higher step; E_i , index potential; K , semiquinone formation constant; M , maximum ratio of semiquinone to total dye. Concentration of dye approximately 0.0005 M.

pH	Buffer	E_m	E_i	$E_m - E_1$ or $E_2 - E_m$	K	M^*
		ms.	ms.			
13.02	NaOH	-208	38	36	16	0.67
12.55	"	-182	41			
11.84	" + KCl	-147	39			
11.51	" + "	-127	40.5			
11.09	Phosphate	-109	32	24	6	0.55
10.04	Dimethyl-glycine (13)	-77	21.7	0	1	0.33
9.67	"	-65	19.0	-16	0.4	0.27
8.51	Veronal	-24	16.2	-37	0.06	0.10
6.23	Phosphate	+95	14.9	-90†	0.001†	0.016†
4.62	Acetate	+197	14.6			
2.03	HCl + KCl	+346	14.7			

* M is calculated according to the equation

$$M = \sqrt{k}/(\sqrt{k} + 2) \quad (1)$$

This equation can be derived more easily than has been done in the previous paper (8). We designate the concentration of the total dye, the reduced form, the semiquinonoid, and the totally oxidized form respectively as a , r , s , t ; then the semiquinone formation constant, k , is defined as $k = s^2/(r \times t)$. The maximum value of s/a exists at 50 per cent oxidation, when $r = t = (a - s)/2$. So, in this case $k = s^2 / \left(\frac{a-s}{2}\right)^2$. This equation, solved

for $s/a = M$, gives directly Equation 1.

† Approximate.

The results are shown in Table I. In Fig. 1, the three normal potentials are plotted against pH. The location of the bends due to the acid dissociation constant of the reduced and of the totally oxidized form, K , and K_t , need no other comment than is given in the previous paper (8). Whereas in all the other quinones re-

ferred to above the existence of an acidic dissociation constant of the semiquinone could not be definitely proved, in this case the constant could be estimated as follows: There is a bend of E_m at

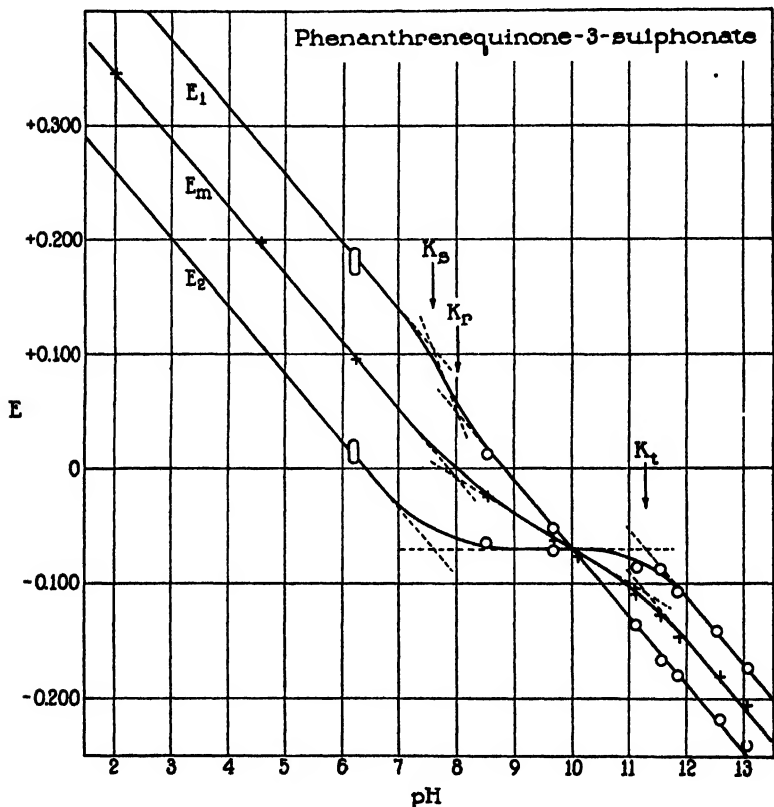


FIG. 1. The three normal potentials, referred to the normal hydrogen electrode at 30°, of phenanthrenequinone-3-sulphonate plotted against pH: E_m , normal potential of the quinone-hydroquinone system; E_1 , that of the semiquinone-hydroquinone system; E_2 , that of the quinone-semiquinone system. K_s , K_r , K_t represent bends due to acidic dissociation constants of semiquinone, hydroquinone, and quinone.

pH 8, due to a K_r . This bend is necessarily accompanied by a still stronger bend in the E_1 curve. So a divergence of the three normal potential curves toward the left-hand side is produced, which would mean that the existence of the semiquinone rapidly

vanishes with decreasing pH. This however can be shown not to be the case. It is true that at $\text{pH} < 6$, under ordinary conditions, the appearance of a semiquinone during the reduction cannot be observed. Yet, on reducing a very concentrated solution of the dye, even at any $\text{pH} < 6$, the color turns from yellow first to a much darker color of a dirty olive-brown shade and then fades out to the practically colorless state of the hydroquinone. The reason why the recognition of the intermediate state is facilitated by working in a high concentration is that the quinonoid form, although intensely yellow, absorbs even in high concentration visible light only of the greater wave-lengths and is quite translucently yellow. The semiquinone is what one might call yellow only in the lowest concentration and appears dark, and almost black with increasing concentration. If the maximum amount of semiquinone should rapidly decrease with decreasing pH, the appearance of the intermediate dark shade should also rapidly vanish from pH 6 downward. This, however, is not the case. This shows that the maximum amount of semiquinone remains constant throughout all pH values < 6 . So there must be in the E_1 and E_2 curves another bend which makes the curves parallel. This bend cannot lie at a $\text{pH} > 8$; for in this case, the three curves would converge immediately to the left side from the crossing point, which can be ruled out by the result of the experiment at pH 8.5. On the other hand, the bend cannot lie at a pH very much smaller than 8, because in this case the mutual distance of three parallel curves would be so great as to indicate a practical non-existence of the semiquinone. So, the possible error will probably lie within a few tenths of a pH unit, if we place the bend at pH 7.5, as has been done in Fig. 1.

In this way it is ascertained that the semiquinone is an acid a little stronger than the hydroquinone. Whether this change in acidic dissociation of the semiquinone is accompanied by a change in color, as it has been shown for the semiquinone of lactoflavin (14), cannot be decided. In the latter case the contrast of color was very distinct, red and green. In the present case we can only tell that the ionized form is brown, and the non-ionized form is such as to give, in a mixture with an enormous excess of the intense yellow quinone, an indistinct dark color, rendering the mixture olive-brown.

The maximum fraction of semiquinone capable of existence in very alkaline solution in equilibrium with the other forms is 0.67. This figure is somewhat higher than for any of the other anionic semiquinones described as yet. It corresponds to an index potential of 40 millivolts and a formation constant of 16. The titration curves, however, are smooth, just as are curves with no step formation, and have no jump in the middle. This, then, is the occasion to discuss, in the next section, an important problem concerning the shape of the curves.

Phenanthrenequinone-2-sulfonate has, according to Fieser (15), a potential 6 millivolts more negative than that of the phenanthrenequinone-3-sulfonate. However, all features regarding the semiquinone formation are so similar in the two cases that there is no need to give the detailed data for the phenanthrenequinone-2-sulfonate.

Problem of the Points of Inflection

Two-step titration curves have a point of inflection at 50 per cent of the total oxidation, just as do one-step titration curves. We shall refer to it as the half-way point of inflection. In addition they may or may not have two more such points, the lateral points of inflection. Whether or not they exist depends on the magnitude of the semiquinone formation constant, k . For very large k they are very distinct and lie at 25 and 75 per cent of oxidation. With decreasing k they become less distinct and approach the half-way point. At a certain threshold value of k they entirely disappear. This threshold value has not been calculated as yet. It had been shown (16) that for $k = 4$ there are no lateral points. If this be the threshold value, our titration curves in very alkaline solutions, for which k was calculated to be 16, should show lateral points; yet they do not. So it is important to calculate the real threshold value in order to justify our interpretations as based on the semiquinone theory.

The calculation, of course, amounts to a double differentiation of the potential function. For this purpose it is advisable to write this function in a shape convenient for differentiation. One suggestion has been made by Elema (17); another will be shown here. It has the advantage, besides, of showing very clearly the symmetry of this function around the mid-point of titration. We

rewrite the formula developed previously (Equation 22 (18)) slightly modified.

$$E = E_m + \frac{RT}{2F} \ln \frac{1+\mu}{1-\mu} + \frac{RT}{2F} \ln \frac{\sqrt{1+\gamma(1-\mu^2)} + \mu}{\sqrt{1+\gamma(1-\mu^2)} - \mu}$$

E_m is the potential in the mid-point of titration. In the previous form of this equation, ξ was defined as the number of equivalents of the oxidizing agent added. It varies during the titration from 0 to 2. In order to emphasize the symmetry of the function around its mid-point, we introduce the variable $\mu = \xi - 1$, shifting the zero point of the function to the mid-point of titration. So, the range of μ is from -1 to $+1$. The dismutation constant κ was defined thus:

$$\kappa = \frac{[\text{reduced form}] \times [\text{oxidized form}]}{[\text{semiquinone}]^2} = \frac{1}{k}$$

k is what we called the formation constant. The definition of γ in the above equation is $\gamma = 4\kappa - 1$. On differentiation, one obtains

$$\begin{aligned} \frac{dE}{d\mu} &= \frac{RT}{F} \times \frac{1}{1-\mu^2} \left(1 + \frac{1}{\sqrt{1+\gamma(1-\mu^2)}} \right) \\ \frac{d^2E}{d\mu^2} &= \frac{2RT}{F} \times \frac{\mu}{\lambda} \times \left[\frac{1}{\lambda} \left(1 + \frac{1}{\sqrt{1+\gamma\lambda}} \right) + \frac{\gamma}{2} \times \frac{1}{(\sqrt{1+\gamma\lambda})^3} \right] \end{aligned}$$

where $\lambda = 1 - \mu^2$. Equating this to 0, we obtain as the first solution $\mu = 0$, or $\xi = 1$. This gives the half-way point, which exists for all values of k . Equating the term in brackets to 0, and putting $\sqrt{1+\gamma\lambda} = q$, we obtain $q^3 + \frac{1}{2}q^2 - \frac{1}{2} = 0$, which gives $q = \frac{1}{2}$.³ Retracing the definition of q back to the former variables, we obtain

$$\xi = 1 \pm \frac{1}{2} \sqrt{\frac{16-k}{4-k}}$$

This equation furnishes the location of the two lateral points. The value of this function has a physical significance only when

³ The other two roots of this equation of third degree are equal to each other; namely, $q = -1$. The two values of ξ corresponding to them are $\xi = 0$, and $\xi = 2$. But at these two values of ξ , the potential is $-\infty$ and $+\infty$ respectively. So the fact that the first derivative is stationary at these two values of ξ does not mean that there are points of inflection.

it is a number which is real and furthermore lies within the physically significant range of ξ , namely from 0 to +2. This condition is fulfilled only when $k > 16$. The threshold value of k , then, is 16. By coincidence, this is just the highest value of k in our experimental curves. So it is in agreement with the semiquinone theory that there are no lateral points of inflection.

It is worth while mentioning that the same formulæ can be applied to the acidimetric titration of a bivalent acid. In this case k represents the ratio of the larger dissociation constant to the smaller one.

SUMMARY

Phenanthrenequinonesulfonate is a substance especially suitable for the solution of a problem which concerns the formation of a semiquinone and is difficult to deal directly with on the biologically occurring quinones. On this example it can be shown that even at such pH at which ordinary methods seem to show the lack of a semiquinone, it still is capable of existence. Furthermore, this example prompts the discussion of a general problem concerning the points of inflection of titration curves.

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THE VERATRINE ALKALOIDS

I. THE DEGRADATION OF CEVINE

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In 1878 Wright and Luff¹ described the separation of the amorphous veratrine obtained from the seeds of *Veratrum sabadilla* into cevadine, $C_{22}H_{49}O_3N$ (veratrine of Merck), veratrine, $C_{27}H_{53}O_{11}N$, and cevadilline, $C_{24}H_{53}O_3N$. Their formula for cevadine which yielded tiglic acid and cevine, $C_{27}H_{45}O_3N$, on saponification holds today. Veratrine or veratridine has recently been shown by Blount² to be $C_{28}H_{51}O_{11}N$ and to be the veratric acid ester of cevine. Cevine itself was first obtained crystalline by Freund and Schwarz³ by the saponification of cevadine with alcoholic potassium hydroxide, and since it separates first as a potassium salt, it has been suggested by Macbeth and Robinson⁴ that a lactone group may be opened. 2 of the oxygen atoms of cevine must be in OH groups, since a diacyl derivative can be formed. The functions of the remaining oxygen atoms have perhaps been indicated to be OH groups by the fact that cevine, according to Freund and Schwarz,⁵ gives 6 moles of CH_4 . Aside from suggestions contained in very early work, nothing of importance seems to have appeared in regard to the actual degradation of cevine until the work of Macbeth and Robinson who reported the isolation from the products of the destructive distillation of cevine with zinc dust and soda lime of a base which they believed to be *l-coniine*. As will be apparent further on, we have not been able to repeat this

¹ Wright, C. R. A., and Luff, A. P., *J. Chem. Soc.*, **33**, 338 (1878).

² Blount, B. K., *J. Chem. Soc.*, 122 (1935).

³ Freund, M., and Schwarz, H. P., *Ber. chem. Ges.*, **32**, 800 (1899).

⁴ Macbeth, A. K., and Robinson, R., *J. Chem. Soc.*, **121**, 1571 (1922).

⁵ Freund, M., and Schwarz, A., *J. prakt. Chem.*, **96**, 236 (1917).

observation. Very recently Blount⁶ has obtained from cevine on selenium dehydrogenation a base, $C_{23}H_{25}N$ (cevanthridine), and apparently a phenol, $C_{17}H_{15}O$ (cevanthrol), the former tetracyclic and the latter possibly a substituted alkyl phenanthrene. Their relationship to the parent alkaloid remains to be determined.

We have ourselves attacked the problem of the degradation of cevine in a number of ways and, since a repetition of its destructive distillation with both soda lime and zinc dust has given in each case a number of simpler substances which will doubtlessly be found to bear simple relationships to the parent structure, these observations will be recorded even though the identities of the substances have not yet been established.

When cevine is heated in an atmosphere of hydrogen with soda lime, water at first distils; but this is followed at higher temperatures by a copious oily distillate. This material, on standing and during the process of fractionation, was found to be comparatively unstable, and its investigation as such proved to be unpromising. All our attempts to fractionate out of the basic portion anything corresponding to coniine were unsuccessful. But comparatively little basic material distilled near the boiling point of coniine and its investigation was without result. It was evident that the mixture consisted largely of unsaturated substances. If the soda lime distillate was directly hydrogenated, it was then found possible to proceed to better advantage. This hydrogenation was accomplished in alcoholic solution containing acetic acid. The hydrogenation mixture was then separated into basic and neutral fractions by steam distillation first from the strongly acidified mixture and then after it had been rendered alkaline. The hydrochloric acid solution of the collected bases gave on concentration a syrupy residue which, in agreement with the observation of Macbeth and Robinson, had imbedded in it small amounts of crystals which were identified as ammonium chloride. This mixture on standing readily deepened in color to a purple.

The liberated bases were then fractionated. Here again a relatively small amount of material distilled up to the boiling point of coniine, and even after refractionation we were unsuccessful in obtaining any evidence of the presence of this base. At

⁶ Blount, B. K., *J. Chem. Soc.*, 124 (1935). Blount, B. K., and Crowfoot, D., *J. Chem. Soc.*, 414 (1936).

a somewhat higher point (about 60–70° at 8 mm.) a fraction was obtained, still an obvious mixture but which gave a crystalline picrate. On recrystallization this salt assumed a form (needles) and melting point (146–150°) which indicated a definite substance. The analytical results agreed with those for a base, $C_{10}H_{19}N$ (or $C_{11}H_{21}N$), and in its reactions it was found to be tertiary. The mother liquors of this picrate yielded a lower melting mixture which crystallized in leaflets and which analysis suggested to be the salt of a base $C_{10}H_{17}N$ or an unsaturated derivative of the above base. This was substantiated by the hydrogenation of the base liberated from the more soluble picrate to a base which now gave a higher melting picrate and was indistinguishable from that which melted at 146–150°.

Nevertheless, the picrate melting at 146–150° was shown to be a mixture, since the free base gave with methyl iodide a quaternary salt which was not homogeneous. From the latter a substance was obtained on recrystallization to constant melting point, which gave figures agreeing with the formula $C_{10}H_{19}N \cdot CH_3I$. This salt was then carried through the steps of exhaustive methylation which gave a mixture of substances as was to be expected. The des-base of the above salt on hydrogenation gave a saturated base which was analyzed as the picrate, $C_{11}H_{23}N \cdot C_6H_5O_7N_3$. From this the methiodide $C_{11}H_{23}N \cdot CH_3I$ was obtained. The des-base from the latter on hydrogenation gave in turn the saturated base which was isolated as the salt $C_{12}H_{27}N \cdot HCl$. This base, while resembling synthetic *n*-decyldimethylamine in some respects, gave a chloroplatinate which melted 20° higher than that of the synthetic base.

This series of steps therefore shows definitely that the base $C_{10}H_{19}N$ from cevine is of dicyclic character with the N atom common to both rings. There are so many possibilities which can be considered in attempting to ascertain the identity of this ring system that suggestions now are premature. However, there comes to mind the possibility of a relationship with lupinane obtained from the alkaloid lupinine which has been shown by the work of Karrer, Canal, Zohner, and Widmer⁷ and the later

⁷ Karrer, P., Canal, F., Zohner, K., and Widmer, R., *Helv. chim. acta*, 11, 1062 (1928).

synthetic work of Clemo and coworkers⁸ to be an octahydro-pyridocoline derivative. The base, $C_{10}H_{19}N$, possibly an octahydropyrrocoline derivative, recently obtained by Clemo⁹ on hydrogenation of an alkaline degradation product of strychnine is described as giving a picrate melting at 147° or approximately the melting point of our picrate from cevine. However, the melting points of the methiodides are not in agreement.

An examination of the neutral fraction of the soda lime distillate of cevine is still in a preliminary phase. The material possesses a characteristic terpene-like odor. On fractionation a partial separation was accomplished. The lower fraction which possessed an odor suggestive of cyclohexanone yielded a semicarbazone melting at $217-219^{\circ}$. The analysis of this substance agreed with the figures for a derivative of a cyclic ketone, $C_7H_{12}O$. Its identity remains to be established. A higher boiling fraction was separated by treatment with sulfuric acid into a hydrocarbon fraction and one from which a crystalline semicarbazone was obtained. Preliminary analytical data in this case indicated the derivative of a dicyclic ketone $C_{11}H_{18}O$.

The origin of these constituents of the neutral fraction is, of course, uncertain but it is possible that such cyclic ketones, if not direct degradation products, may originate in cyclization of the salts of dibasic acids produced by alkali cleavage of the alkaloid molecule. Thus, there is a possibility that cevine possesses a tertiary dicyclic base which may be joined with another ring system. It is hoped that further work on the identification of these degradation products will aid in interpreting the parent structure.

Finally, a preliminary study has been made also of the distillation of cevine with zinc dust. Here the course of the decomposition was found to be different from that with soda lime. The basic fraction proved to be a complex mixture of strong and weak bases. One of these, present in small amount, was readily separated from the others by the fact that on distillation of an ether solution of the bases it was carried off with the ether vapor and readily gave a picrate which still awaits identification.

⁸ Clemo, G. R., and coworkers, series of articles in *J. Chem. Soc.* (1928-36).

⁹ Clemo, G. R., *J. Chem. Soc.*, 1695 (1936).

The latter, which melted at 178–180°, yielded analytical figures agreeing with the formula $C_7H_{15}N \cdot C_6H_5O_7N_3$. The base proved to be tertiary and contained an N-methyl group. A mixed melting point with the picrate of N-ethyl piperidine showed a depression. After further fractionation of the mixture of bases which possessed a strong odor of pyridine bases a number of picrates were obtained. One of these which crystallized in leaflets melted at 132–133° and appeared to be homogeneous. Its analysis indicated it to be the salt of a pyridine base $C_8H_{11}N$. Its identity has not yet been determined. In the higher boiling fractions an apparently homologous base was contained, since the analysis of its picrate indicated a formula $C_9H_{13}N$. We were unable again to obtain any evidence of the presence of coniine in the zinc dust distillate.

There is still in progress a further study of the fractionation and identities of the degradation products which have been obtained from cevine.

EXPERIMENTAL

Soda Lime Distillation of Cevine

An intimate mixture of 10 gm. of cevine and 90 gm. of soda lime was heated in a glass vessel immersed in a sodium-potassium-nitrate bath with a slow stream of hydrogen passing through the apparatus during the entire operation.

The vessel consisted of an elongated Pyrex bulb fitted at the front end with a glass tube for the hydrogen intake and at the exit end with a longer V-shaped tube with a bulb blown at the lower part. The latter was immersed in ice for condensation of the distillate. The escaping gases were passed through a train of dilute HCl wash bottles. During the first 10 minutes the bath was raised to about 280°, when mainly water had distilled. Above this an appreciable distillation of oil occurred, at first yellow but gradually deepening to a red-brown in color. The temperature was kept for several hours at 300° and finally raised to 335–345° for about 3 more hours, during which a dark colored, more viscous resin distilled.

The distillate was transferred with alcohol into a hydrogenation apparatus, acidified with acetic acid, and then shaken with 0.1 gm. of the platinum oxide catalyst of Adams and Shriner. After

reduction of the catalyst a prompt further absorption of hydrogen occurred which amounted on the average to about 100 to 120 cc. in the 1st hour and then became more gradual. On continuing for about 18 hours, a total of about 200 cc. was absorbed.

For the next step, experiments were combined representing a total of 70 gm. of cevine. The filtrate from the catalyst was strongly acidified with HCl and then distilled with steam. The alcohol was followed by a colorless oil which continued to go over even after 1 hour. This fraction which will be called the neutral fraction will be discussed below.

The residual solution was then made strongly alkaline with NaOH and the steam distillation was resumed. The distillate was collected in a flask followed by a series of bottles containing 10 per cent HCl. Here again several hours were required for the complete distillation of the less volatile basic material. The combined distillates were then concentrated *in vacuo* to dryness, during which the solution assumed a red-purple color. The residue was decomposed in the cold with 25 per cent KOH. The liberated basic oil (about 7 cc.) was pipetted off and dried over KOH. It was then fractionated.

All attempts to fractionate this material showed that it was a very complex mixture. Very meager distillation began at about 140° at 760 mm. but the temperature rose rapidly above 170°, with the bath reaching 220°. This fraction, an obvious mixture, amounted to only 0.36 gm. All later attempts to fractionate it further or to prepare crystalline derivatives were fruitless.

Distillation was then resumed at low pressure. At 8 mm. a fraction (B) was collected, which rose steadily from 65–100°. The yield was 2.2 gm. It was found advantageous to convert this fraction, without further fractionation, directly into the picrate as given below. In similar experiments where such refractionation was carried out, a main portion appeared to boil at 8 mm. and 60–70° and gave the following analytical figures.

$C_{10}H_{19}N$.	Calculated.	C 78.35, H 12.50
$C_9H_{17}N$.	"	" 77.62, " 12.31
	Found.	" 77.85, " 12.44
	"	" 77.72, " 12.56

The picrate obtained from such material, however, proved to be a mixture.

A third fraction (C) was then collected, which distilled at 8 mm. from 100–140°. The yield was 2.8 gm. On refractionation an appreciable proportion appeared to boil fairly constantly at 105–109° at 8 mm. and formed a rather viscous liquid. No crystalline derivative could be obtained from this fraction.

$C_{10}H_{19}ON$. Calculated. C 70.94, H 11.32, N 8.28
 Found. " 71.00, " 11.84, " 9.04

Fraction B (2.2 gm.) was treated with 3.3 gm. of picric acid in 15 cc. of alcohol. Prompt crystallization of a mixture of leaflets and needles occurred on cooling. After collection and washing of the substance with ether, its melting point was not sharp; it melted at 85–120°. After repeated recrystallization from alcohol only needles remained, which began to sinter at 142° and mostly melted at 148–150°. The yield was 0.9 gm. The analytical results on samples obtained on different occasions fluctuated between those required for the salt of a $C_{10}H_{19}N$ and a $C_{11}H_{21}N$ base.

$C_{10}H_{19}N \cdot C_6H_3O_7N_3$. Calculated. C 50.23, H 5.80, N 14.66
 $C_{11}H_{21}N \cdot C_6H_3O_7N_3$. " " 51.49, " 6.10, " 14.14
 Found. " 50.87, " 6.09, " 14.47
 " 51.20, " 5.98

The NCH_3 determination was negative.

From the recrystallization mother liquors of the previous picrate a more soluble picrate was obtained, which crystallized as leaflets. This appeared to be still a mixture, since repeated recrystallization did not sharpen the melting point. It softened above 110° and appeared to melt mostly at 118–120°, but a small amount of crystals persisted under the polarizing microscope until 136° was reached.

Analysis of such material gave the following figures.

$C_{10}H_{17}N \cdot C_6H_3O_7N_3$. Calculated, C 50.50, H 5.30; found, C 50.32, H 5.63

The low hydrogen figures suggested unsaturation. When the dilute acetic acid solution of the base liberated from the low melting picrate was hydrogenated, absorption was noted and about 50 per cent was recovered after conversion into the picrate. The latter now melted at 145–149°.

The hydrochloride obtained from the picrate partly crystallized on drying down its solution, but was too soluble for manipulation. The unhomogeneous nature of the base was shown on conversion to the methiodide as follows.

The Methiodide—1.07 gm. of the picrate were treated with excess 10 per cent hydrochloric acid and the picric acid liberated was extracted with ether. After concentration of the acid solution *in vacuo* to dryness and redissolving the residue in a small volume of water, the base was liberated with excess potassium hydroxide and extracted with ether. The dried ether extract was treated with excess methyl iodide and allowed to stand overnight at room temperature. The crystalline material was collected with ether. Its melting point was not sharp, 168–180°. Upon recrystallization from acetone 0.35 gm. of material was obtained, which still did not give a sharp melting point. Under the microscope it began to melt at 200° but the last crystal did not disappear below 230°. Further recrystallization did not change the melting point. The methiodide in alcoholic solution showed no optical activity.

$C_{10}H_{11}N \cdot CH_3I$. Calculated, C 44.73, H 7.70; found, C 45.07, H 7.54

Degradation of the Methiodide—0.345 gm. of the methiodide was dissolved in 2 cc. of methyl alcohol and treated with silver oxide suspended in methyl alcohol until the filtrate gave no further test for halogen. It gave no test for dissolved silver. The clear, colorless filtrate was carefully fractionated, at first under atmospheric pressure until all of the methyl alcohol had distilled and then under 15 mm. pressure. Most of the residual oil distilled when the oil bath temperature was 90–100°, but everything distilling up to a temperature of 130° was collected in the distillate. The distillate was immediately transferred to the hydrogenation apparatus with 3 cc. of glacial acetic acid and 25 mg. of Adams and Shriner's catalyst were added. After 2 hours no more hydrogen was absorbed. The amount absorbed was about 75 per cent of the theoretical for 1 mole of hydrogen based on the methiodide used. The catalyst was filtered off and the filtrate was treated with excess hydrochloric acid. The solution was then evaporated to dryness *in vacuo*. It could not be made to crystallize. A weighed portion of the hydrochloride was dissolved in a few drops

of water and the base was liberated with solid potassium hydroxide. The ether extract after drying over potassium carbonate was treated with a calculated amount of picric acid. After cautious evaporation of the solvent, the residue was crystallized from ethyl alcohol. Upon recrystallization from alcohol the picrate formed sharply crystalline yellow rhombs which melted at 138–140°.

$C_{11}H_{22}N \cdot C_6H_3O_7N_3$. Calculated, C 51.23, H 6.58; found, C 51.46, H 6.70

The free base was liberated as above from a portion of the hydrochloride and the ether solution was treated with excess methyl iodide. After standing overnight at room temperature the crystalline material was collected. Upon recrystallization from acetone it melted at 248–250°.

$C_{11}H_{22}N \cdot CH_3I$. Calculated, C 46.23, H 8.44; found, C 46.23, H 8.14

The free base obtained from the above picrate gave the same methiodide upon treatment with methyl iodide and recrystallization from acetone.

100 mg. of this methiodide were dissolved in 2 cc. of methyl alcohol. Silver oxide suspended in methyl alcohol was added until the filtrate gave no further test for halogen. It also gave no test for silver. The filtrate was fractionated at first up to an oil bath temperature of 100° at atmospheric pressure and then under 15 mm. pressure. Most of the residual oil distilled at 90–100°, although the temperature of the oil bath was raised to 130°. The colorless distillate was transferred to the hydrogenation apparatus with 3 cc. of glacial acetic acid and 25 mg. of Adams and Shriner's catalyst were added. In 2.5 hours absorption of hydrogen stopped practically at the theoretical point for 1 mole of hydrogen. The filtrate from the catalyst was treated with excess hydrochloric acid and evaporated to dryness *in vacuo*. The residue was carefully dried. It weighed 62 mg. Upon recrystallization from acetone 13 mg. of broad, thin leaves were obtained which melted with sublimation at 185–193°.

$C_{11}H_{21}N \cdot HCl$. Calculated, C 65.26, H 12.76; found, C 64.90, H 12.57

3.4 mg. of the hydrochloride were dissolved in a small volume of 10 per cent hydrochloric acid and a solution of 10 per cent

chloroplatinic acid was added until no further precipitation occurred. 4.2 mg. of crystalline material which melted at 113–116° were collected with 10 per cent hydrochloric acid. Upon recrystallization from dilute hydrochloric acid 1.7 mg. of needles remained, which melted after preliminary softening at 118–120°.

$(C_{12}H_{17}N)_2 \cdot H_2PtCl_6$.	Calculated.	C 36.91,	H 7.23,	Pt 25.02
	Found.	" 37.21,	" 7.12,	" 24.63

The picrate of the base proved to be very soluble.

For comparison, *n*-decyl bromide, kindly placed at our disposal by Professor E. Emmet Reid, was converted into the dimethyl-*n*-decylamine for comparison. The chloroplatinate prepared from this formed needles which melted at 97–98°. Found, C 37.11, H 7.14, Pt 24.83. This substance, when mixed with the above chloroplatinate, showed no definite melting point depression and appeared to have the same crystalline form.

The Neutral Fraction—The neutral fraction from a number of experiments (from about 180 gm. of cevine) was well diluted and extracted with ether. The ether extract was washed repeatedly with water to remove alcohol and then shaken with 1:1 HCl to remove any weakly basic material. It was then washed with water and dried. The ether was then slowly distilled off until the residue was about 25 cc. in volume and still contained ether and low boiling solvents. This residue was placed in a small flask with a fractionating column 20 cm. in length and fractionated. Ether was first removed and then in Fraction 1 material was collected boiling up to 50° under 25 mm. pressure. Fraction 2 distilled up to 98° under 25 mm. pressure. Fraction 3 distilled up to 105° under 10 mm. pressure. Fraction 4 distilled up to 105° under 2 mm. pressure. The residue in the flask was then transferred to a microstill¹⁰ and material (Fraction 5) collected up to an oil bath temperature of 165° and under 0.4 mm.

The lower boiling fractions had an odor resembling cyclohexanone, while the higher boiling fractions had a terpene-like odor. Fraction 2 was treated with an equal weight of semicarbazide hydrochloride, 1.2 gm. of sodium acetate, and 8 cc. of water. After standing 24 hours at room temperature, the oil had become

¹⁰ Craig, L. C., *Ind. and Eng. Chem., Anal. Ed.*, **8**, 219 (1936).

partly crystalline. It was treated with an equal volume of ether and the solid filtered off. It weighed 0.3 gm. After recrystallization three times from methyl alcohol it melted at 217–219°.

$C_8H_{11}ON_2$. Calculated. C 56.80, H 8.93, N 24.85
Found. " 56.79, " 8.68, " 25.02

Fraction 3 was dissolved in 3 cc. of a low boiling petroleum ether (36–41°) and was shaken out with 5 cc. of cold concentrated sulfuric acid. It was washed with fresh sulfuric acid and set aside. The sulfuric acid layer was diluted with ice and then water, and the insoluble oil was extracted with petroleum ether. The petroleum ether extract was dried over potassium carbonate and fractionated. About 150 mg. of oil distilled at an oil bath temperature of 150–160° under 25 mm. pressure. This fraction gave a crystalline semicarbazone when treated as above.

Fraction 4 was treated as in the case of Fraction 3. Upon drying with potassium carbonate the sulfuric acid-insoluble fraction contained in the petroleum ether extract and fractionating, a colorless oil (500 mg.) with a faint petroleum odor was obtained which distilled at an oil bath temperature of 120–130° under 0.2 mm. pressure. Analytical data showed this oil to be a hydrocarbon. Found, C 89.60, H 10.18.

The sulfuric acid-soluble fraction after extraction with ether, drying, and fractionation gave 0.6 gm. of oil boiling at an oil bath temperature of 120–140° which had a strong terpene-like odor. Upon treatment with semicarbazide hydrochloride as above, it gave a crystalline semicarbazone melting at 160–170°. Preliminary analytical data indicated the semicarbazone of a ketone $C_{11}H_{18}O$.

$C_{12}H_{21}ON_2$. Calculated, C 64.67, H 9.54; found, C 65.03, H 9.06

Zinc Dust Distillation of Cevine

15 gm. of cevine were intimately mixed with 300 gm. of zinc dust and heated in the apparatus described in the case of the soda lime fusion, during which a slow stream of hydrogen was passed through. On heating above 250°, water appeared in the condenser bulb and this continued on further heating to 300°. A light yellow, easily volatile oil then began to appear. After 2 hours

the bath was raised to 340°, at which point during the next few hours a viscous brown oil accumulated. When a larger run was made, *e.g.* 45 gm. of cevine, it was necessary to heat the bath about 20° higher.

The distillates from 60 gm. of cevine were dissolved in about 100 cc. of alcohol and after acidification with acetic acid hydrogenated with 0.2 gm. of Adams and Shriner's catalyst. Absorption of hydrogen was at first rather rapid and amounted, exclusive of that taken by the catalyst, to 175 cc. in 35 minutes. 0.3 gm. more catalyst was then added and the hydrogenation was allowed to continue for 18 hours. By this time the absorption was roughly 1 liter. Although it continued, the hydrogenation was interrupted. The resulting solution was separated into basic and neutral fractions as in the case of the soda lime experiments. The mixture of bases which were steam-distilled into dilute hydrochloric acid gave on concentration a dark colored syrup of mixed hydrochlorides. When decomposed with alkali, an appreciable volume of dark colored basic oil was liberated. To facilitate separation, the latter was extracted with about 50 cc. of ether and the solution was dried over KOH. The ether was then slowly and carefully distilled. The distillate (Fraction A) had a definite basic odor, suggesting a piperidine derivative. It was treated with a slight excess of ethereal picric acid. A turbidity followed by crystallization occurred, which was increased by concentration.

The yield was 45 mg. On recrystallization from alcohol, the picrate formed needles which melted at 178–180°. It was shown to be a tertiary base.

$C_7H_{11}N \cdot C_6H_5O_7N_3$.	Calculated.	C 45.59, H 5.30, CH ₃ 4.39
$C_7H_{11}N \cdot C_6H_5O_7N_3$.	"	" 45.86, " 4.74
	Found.	" 45.88, " 4.95, CH ₃ 3.36

On resumption of the distillation of the crude mixture of bases a dry ice trap was placed at the end of the receiver to catch any escaping bases. A second fraction in very small amount slowly accumulated in the condenser as the bath was raised to 175° at 760 mm. The inside thermometer barely reached 127°.

This fraction (B), which smelled strongly of pyridine bases, also yielded a crystalline picrate which was an obvious mixture.

The distillation was now resumed at 7 mm. A pyridine base-smelling fraction was collected from 30–50°, mostly at 40–43°. For Fraction C the yield was 0.55 gm. A fourth fraction was collected at 7 mm. between 50–75°. For Fraction D the yield was 0.35 gm. Finally, a last fraction was collected at 7 mm. and 75–100°. For Fraction E the yield was 0.32 gm.

Both Fractions C and D readily gave crystalline picrates, whereas Fraction E gave a much smaller yield. Fraction C was treated in alcoholic solution with 1.1 gm. of picric acid. On cooling it formed a thick mass of leaflets. 0.4 gm. was obtained, which on recrystallization from alcohol melted at 128–133°. This was scarcely improved by recrystallization.

$C_8H_{11}N \cdot C_6H_3O_7N_3$. Calculated, C 47.98, H 4.03; found, C 47.56, H 3.93

Fraction D similarly gave 0.54 gm. of picrate, also leaflets, from alcohol which melted at 132–133°. Found, C 48.15, H 3.93. Fraction E gave 0.2 gm. of picrate from alcohol, which partly melted above 131° but did not clear till 142°. Recrystallization from acetone gave again leaflets with melting point unchanged.

$C_8H_{11}N \cdot C_6H_3O_7N_3$. Calculated, C 49.43, H 4.43; found, C 49.18, H 4.14

The dry ice trap, when rinsed out with ether after the distillation, gave a copious crystalline picrate with ethereal picric acid. Preliminary study of this material showed it to be a mixture.

There has not been opportunity to investigate as yet the neutral fraction obtained from the zinc dust distillation.

3,5-Dinitrobenzoyl-d-Coniine—*d*-Coniine was readily acylated with 3,5-dinitrobenzoyl chloride in benzene solution by the Schotten-Baumann reaction. The derivative readily crystallized from alcohol as needles which melted at 108°.

$C_{18}H_{21}O_5N_5$. Calculated, C 56.04, H 5.96; found, C 56.17, H 6.09

Addendum—We have recently confirmed the presence of coniine in the soda lime distillate in very small amount by isolation as the 3,5-dinitrobenzoyl derivative. The base, $C_7H_{11}N$, produced by zinc dust distillation is apparently active *N*-methyl- β -pipecoline. β -Pipecoline, itself, has also been isolated. Details will follow.

THE SAPOGENINS OF POLYGALA SENEGA

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Although a number of references to the saponin of *Polygala senega* or senega-root are to be found in the older literature, it has not been possible to uncover anything of importance relating to its structural chemistry. The more recent work of Wedekind and Krecke¹ represents apparently the first real attempt in this regard. The latter, working with a saponin obtained as such from E. Merck, Darmstadt, found it to yield on hydrolysis a genin, senegenin, to which the formula $C_{28}H_{48}O_8$ was assigned. It was shown to be dibasic. The presence of two OH groups was indicated by the formation of a diacetyl derivative, the nature of which was concluded from its titration. A dimethyl ester (m.p. 206–208°) was also mentioned, but without analytical data.

Our own interest in the subject began before the recent developments in the chemistry of the sapogenins in which the method of dehydrogenation has so helped to sharpen their division into members of the triterpene and sterol groups. It was of importance then to determine in which of these categories the polybasic sapogenins would fall, if in either. Recently it has been possible to resume this study, but in the meantime the interesting series of papers by Wieland and coworkers² on quinovic acid, another polybasic sapogenin, has appeared and, as will become apparent, senegenin has been found to belong apparently in the same general group.

The saponin studied by us was prepared in this laboratory from

* Holder of a fellowship from the Swiss-American Student Exchange.

¹ Wedekind, E., and Krecke, R., *Ber. chem. Ges.*, **57**, 1118 (1924).

² Wieland, H., Hartmann, A., and Dietrich, H., *Ann. Chem.*, **522**, 191 (1936), and previous papers by Wieland and coworkers.

commercial senega-root by extraction with alcohol. The partly purified saponin yielded on hydrolysis first a gelatinous prosapogenin which on further treatment with acid gave a sapogenin mixture. It soon became apparent that a number of substances were produced, and considerable study was required to find a suitable method of isolation and purification. Two of these were obtained in pure form and are the subject of the present report. The results obtained differed materially from those of Wedekind and Krecke, so that either their material was of different origin or it was not homogeneous.

One of our substances, which will be called *senegenin*, melted at 290–292°, was separated because of its lower solubility, and gave on analysis figures which agreed with the formula $C_{30}H_{44}O_8$ or $C_{30}H_{44}O_8$. Titration showed it to be dibasic and on heating with alkali a third equivalent was consumed, undoubtedly owing to cleavage of a lactone group. Although it resisted well the action of acids, it could not be recovered from the saponification mixture because of deep seated alteration. An amorphous *dimethyl ester* was prepared with diazomethane and in this derivative, although the lactone group was still readily saponified, the ester groups proved to be very resistant to saponification. Senegenin readily formed a *diacetyl derivative*. A high melting by-product of unexplained nature was simultaneously produced. Senegenin could not be hydrogenated and it is not certain whether a very weakly positive test obtained with tetranitromethane indicates unsaturation. The functions of the 8 oxygen atoms have been explained, since 4 are contained in two COOH groups, 2 in a lactone group, and 2 in two OH groups. From the analytical figures it is not possible to decide directly between the two possible formulas $C_{30}H_{44}O_8$ and $C_{30}H_{44}O_8$. In the former case, if no double bond occurs in the sapogenin, it would be pentacyclic, and in the latter case tetracyclic; but if the color with tetranitromethane means unsaturation, then it would be tetracyclic even in the former case.

In the more soluble sapogenin fraction crystalline material was contained from which it was possible to isolate a second sapogenin derivative which analysis indicated to possess the formula $C_{31}H_{50}O_6$ or $C_{31}H_{48}O_6$. It was found to be a monobasic acid. Contrary to senegenin, however, no lactone group could be detected on boiling with dilute alkali. However, further investigation showed that

the substance contained an ethyl group and was the monoethyl ester of a *dibasic acid*, $C_{29}H_{46}O_6$ ($C_{29}H_{44}O_6$). The latter was obtained from the ester by vigorous treatment with alkali. The ethyl ester group undoubtedly was formed by partial esterification of the dibasic acid during the hydrolysis of the saponin in dilute alcohol. The neutral mixed *methyl ethyl ester* was amorphous and, like senegenin ester, proved to be very resistant to saponification. This sapogenin also gave a *diacetyl derivative*. As in the case of senegenin diacetate, a small amount of a high melting by-product, likewise of unexplained nature, was formed. A *di-p-bromobenzoate* of the acid ester was also prepared. The acid $C_{29}H_{46}O_6$, therefore, is a dihydroxydibasic acid. The acid ester gave a positive test with tetranitromethane in chloroform solution and more pronounced than in the case of senegenin, but no double bond could be directly detected by hydrogenation. Although not definite, the available data as in the case of senegenin might appear to favor a tetracyclic structure.

The thought at once occurred of a possible relationship between senegenin and the second sapogenin. Attempts to prepare the latter by the continued action of alcoholic or dilute alcoholic acid on senegenin were unsuccessful. However, it is still possible that senegenin and its companion product do not occur as such but as a common precursor, perhaps of tribasic character ($C_{30}H_{48}O_6$), and that during the hydrolysis a labile carboxyl and a labile hydroxyl group are protected by lactonization (perhaps after preliminary stereoisomerization) with the formation of the dibasic lactone acid, senegenin. On the other hand, a portion of such a hypothetical acid, $C_{30}H_{48}O_6$, could lose both CO_2 and water to give the unsaturated dibasic acid $C_{29}H_{46}O_6$. Further work will be necessary to test such a suggestion.

The dehydrogenation of senegenin and the companion monoethyl ester was also studied. In these cases the dehydrogenation with selenium proceeded smoothly and with no evidence of the formation of naphthalin hydrocarbons as usually occurs with triterpene derivatives. In the case of senegenin, the largest fraction yielded a hydrocarbon which crystallized as lustrous leaflets and melted at 246.5° . The analysis agreed with the formulation $C_{29}H_{30}$ or $C_{29}H_{28}$. Its solubilities and other properties resembled closely those of chrysene. These properties suggest

that it is perhaps a homologue of the latter. A hydrocarbon $C_{25}H_{40}$ with a melting point of 245° was described by Ruzicka and Ehmann³ as produced in small amount on dehydrogenation of hederagenin. A sample of this substance kindly sent to us by Professor Ruzicka resembled closely our hydrocarbon and gave no melting point depression. There is therefore a possibility that they are identical. However, in the case of senegenin it is produced in much larger amount and in fact appears to be a principal dehydrogenation product. This was especially evident in the case of the dehydrogenation of the companion sapogenin.

The highest boiling fraction yielded in very small amount a hydrocarbon which melted at 298° and gave analytical figures corresponding to a trimethylpicene, $C_{25}H_{40}$. It showed no depression in melting point when mixed with the trimethylpicene from oleanolic acid, for which we are also indebted to Professor Ruzicka. The ultraviolet absorption curves obtained with both were indistinguishable (Fig. 1).

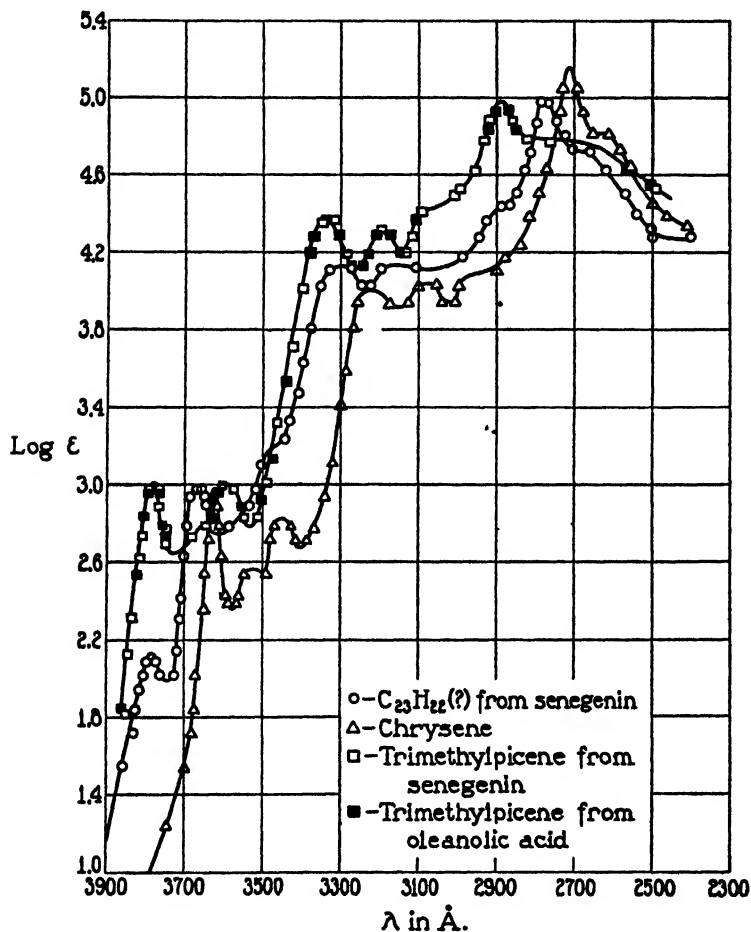
The dehydrogenation of the companion sapogenin appeared to proceed even more smoothly than in the case of senegenin. Hydrocarbon fractions which amounted to at least 25 per cent of the sapogenin used consisted principally of the chrysene hydrocarbon which melted at 246.5° and was indistinguishable from the substance obtained from senegenin. Finally, again a very small amount of the $C_{25}H_{40}$ hydrocarbon (trimethylpicene) was isolated, which melted at 297.5° and again proved to be apparently identical with the second hydrocarbon from senegenin. The trimethylpicene is a substance which Ruzicka and coworkers⁴ have obtained on dehydrogenation of a number of triterpenes and its formation has therefore given weight to the interpretation of these substances as picene derivatives.

It is premature to attempt to present a possible structure for the sapogenins of senega-root, and the question must be left open as to whether they are tetracyclic chrysene derivatives or pentacyclic picene derivatives. It is of interest that Wieland, Hartmann, and Dietrich² on dehydrogenation of quinovic acid

³ Ruzicka, L., and Ehmann, L., *Helv. chim. acta*, **15**, 447 (1932).

⁴ Ruzicka, L., Brüttinger, H., Egli, R., Ehmann, L., Furter, M., and Hösl, H., *Helv. chim. acta*, **15**, 431 (1932). Ruzicka, L., Hösl, H., and Ehmann, L., *Helv. chim. acta*, **17**, 442 (1934).

(pyroquinovic acid) obtained upwards of about 10 per cent of a hydrocarbon, $C_{23}H_{20}$, apparently identical with Ruzicka's trimethyl-



ϵ is the molecular extinction coefficient;
chloroform, the solvent

FIG. 1

picene, and a smaller yield of a second hydrocarbon, C_nH_n , melting at 202° . There was no mention, however, of a substance corresponding to our $C_{23}H_{22}$ hydrocarbon, although it is possible that

the $C_{25}H_{42}$ hydrocarbon may also be a chrysene derivative and a homologue of our substance. There is undoubtedly a significance in the fact that senegenin should give principally a chrysene hydrocarbon, while quinovic acid gives principally a picene derivative. This might perhaps support the tetracyclic nature of the former. However, many more data must be obtained before such a conclusion is justified, and we hope in further work to go more critically into this question.

We are especially indebted to Dr. Alexandre Rothen of this Institute for the ultraviolet absorption spectra determinations (as well as the plotting of the curves) which were generously carried out by him.

EXPERIMENTAL

The senega-root (*Polygala senega*) employed in our studies was the commercial material. A number of samples obtained at separate times from different crude drug houses gave essentially the same results. For the preparation of the crude saponin the following method was employed.

2 kilos of the ground root were refluxed in 6 liters of 95 per cent alcohol for 3 hours and then filtered hot. The extraction was repeated twice, each time with 4 liters of alcohol. The combined alcoholic filtrates were kept at 0° for 48 hours during which a copious precipitate of crude saponin mixed with fats separated. The collected material was dried and then thoroughly defatted in a Soxhlet apparatus with ether. The crude saponin was dissolved in about 30 parts of alcohol and refluxed with bone-black and then filtered hot. After standing 24 hours at 0° the colorless, amorphous saponin was collected. More material was recovered from the mother liquor by concentration to one-third volume. The two fractions were joined for further purification by reprecipitation from alcohol.

From 45 kilos of senega-root 950 gm. of crude senegin were thus obtained. On repeated fractionation from alcohol no crystalline saponin could be obtained. On rapid heating, it colored between 220–230° and decomposed towards 250°. The degree of homogeneity of this material could not be decided. All fractions ob-

tained on attempting repeated fractionation gave a similar sapogenin mixture on hydrolysis.

Hydrolysis of Senegin—100 gm. of twice "recrystallized" senegin were dissolved in 800 cc. of hot 5Q per cent alcohol and then treated with 200 cc. of HCl (sp. gr. 1.19). A voluminous brown precipitate of prosapogenin soon began to form on further heating. For completion, the mixture was refluxed for 30 minutes and then allowed to cool. The prosapogenin precipitate was centrifuged from a dark colored mother liquor which was then decanted. The precipitate was resuspended in 30 per cent alcohol and recentrifuged. This process was then repeated several times with water. The prosapogenin without drying was further hydrolyzed by refluxing in a mixture which consisted of 6 parts of alcohol, 2 of water, and 2 of concentrated HCl based on the weight of the original saponin. After 2 hours the solution was rapidly filtered from a small amount of flocculent material and the heating resumed for another 2 hours, during which crystalline senegenin separated. The mixture was cooled to 60° and filtered at this temperature.

The collected sapogenin melted at 280–290° and was quite pure. The mother liquor left overnight at 0° gave a good amount of a mixture which melted at 230° after preliminary sintering. When the filtrate from this material was heated to boiling and carefully treated with water and cooled, more crystalline material was obtained; but as the process was repeated, the final precipitates became amorphous. The sapogenin fractions were carefully washed in each case and recrystallized from dilute alcohol. In this manner there were obtained from 920 gm. of senegin 35 gm. of senegenin, 74 gm. of mixed crystals, and 40 gm. of amorphous sapogenin.

Senegenin—The above high melting, first sapogenin fraction was purified by dissolving in hot 60 per cent alcohol and then slowly boiling the solution down. The first precipitate containing a sparingly soluble impurity was filtered off hot and discarded. The filtrate was concentrated further until most of the senegenin separated as needles and prisms. It was collected while hot. The mother liquor contained mostly low melting sapogenin mixtures.

Pure senegenin melts at 290–292°.

$$[\alpha]_D^{25} = +19^\circ \text{ (} c = 0.84 \text{ in 95\% alcohol)}$$

It is easily soluble in alcohol, acetone, and acetic acid and practically insoluble in chloroform and benzene. In aqueous alcohol it is more soluble than the companion sapogenin, but mixtures of the two form mixed crystals which are extremely difficult to separate by recrystallization alone.

For analysis three different preparations of senegenin were repeatedly recrystallized from alcohol and dried at 120° and low pressure.

$C_{30}H_{46}O_8$.	Calculated.	C 67.37,	H 8.68
$C_{30}H_{46}O_8$.	"	" 67.63,	" 8.33
	Found. (a)	" 67.51, 67.44,	" 8.56, 8.58
	" (b)	" 67.20,	" 8.50
	" (c)	" 67.45, 67.34,	" 8.52, 8.62

9.455 mg. of substance dissolved in 1 cc. of alcohol were titrated against phenolphthalein with 0.1 N NaOH. Calculated for 2 equivalents, 0.354 cc.; found, 0.335 cc. 3 cc. of alkali were then added and after boiling for 2 hours the mixture was titrated back. Calculated for 1 equivalent, 0.177; found, 0.172 cc.

11.820 mg. of substance on direct titration required 0.416 cc. Calculated for 2 equivalents, 0.454 cc. On saponification, as given above, an additional 0.212 cc. of alkali was consumed.

Senegenin could not be recovered from the above saponification mixtures. The product proved to be an amorphous, apparently dibasic decomposition product. Its analysis (C 69.1, H 8.8) suggested loss of CO_2 after saponification of the lactone group.

Senegenin in acetic acid solution gives a very weak yellow color with tetranitromethane. Although altered by alkalies, it is quite stable on boiling with dilute acids. On heating in a current of nitrogen, 2 moles of CO_2 are split off.

The Dimethyl Ester—A suspension of senegenin in ether was esterified with diazomethane. The ester could not be obtained crystalline. The amorphous material obtained by careful dilution of the methyl alcoholic solution was dried for analysis at 80° and low pressure.

$C_{32}H_{50}O_8$.	Calculated.	C 68.28, H 8.97, OCH ₃ 11.04
$C_{32}H_{50}O_8$.	"	" 68.64, " 8.64, " 11.08
	Found.	" 68.22, " 8.94, " 10.51

The ester gave a weak yellow color with tetranitromethane. The ester groups proved to be difficult to saponify since only 1 equivalent of alkali was consumed (by the lactone group) on saponification.

12.255 mg. of substance were refluxed in 2 cc. of alcohol and 3 cc. of 0.1 N NaOH for 2 hours and titrated back against phenolphthalein. Calculated for 1 equivalent, 0.218 cc.; found, 0.207 cc.

Senegenin Diacetate—0.2 gm. of senegenin was heated at 100° for 1 hour with a mixture of 2 cc. of acetic anhydride and 0.1 gm. of fused sodium acetate. On decomposition of the mixture with water, a colorless resin deposited. After decantation from the latter and washing, the resin was dissolved in a small volume of alcohol. On careful dilution a sandy, crystalline deposit formed in small amount, which will be described below. The mother liquor was evaporated to dryness. The resinous residue on treatment with a small volume of ether crystallized as flat prisms. It was collected with ether in which it was appreciably soluble. It softened above 260° and melted with effervescence at 270°.

$C_{24}H_{30}O_{10}$	Calculated.	C 65.98, H 8.15
$C_{24}H_{30}O_{10}$	"	" 66.19, " 7.85
	Found. (a)	" 65.80, " 8.08
	" (b)	" 66.21, " 8.07

13.092 mg. of substance were titrated with 0.1 N NaOH against phenolphthalein. Calculated for 2 equivalents, 0.424 cc.; found, 0.395 cc. After addition of 3 cc. of 0.1 N NaOH and refluxing for 2 hours, the mixture was again titrated. Calculated for 3 equivalents (lactone and two acetyl groups), 0.636 cc.; found, 0.671 cc.

The above sparingly soluble by-product of the acetylation was recrystallized by concentration of its hot alcoholic solution. It formed minute prisms which melted at 313° after preliminary sintering. Found, C 66.76, H 7.90.

The Dihydroxydicarboxylic Acid Monoethyl Ester, $C_{21}H_{30}O_6$ —20 gm. of the sapogenin mixture, which represented the second crystal crop from the hydrolysis of senegin, were dissolved in a liter of alcohol. To this were added 8 gm. of NaOH dissolved in 1 liter of water. The mixture was refluxed for 2 hours. The residual high melting sapogenin was thus decomposed. The hot solution was then acidified to Congo red with 10 per cent HCl. On cooling,

the new sapogenin derivative crystallized as long needles. For purification it was suspended in 400 cc. of water and treated, with stirring, with 130 cc. of 20 per cent Na_2CO_3 solution. The sparingly soluble sodium salt separated. After standing the salt was collected, dissolved in alcohol, and decomposed with an excess of HCl . When the hot solution was treated with an equal volume of water and allowed to cool, the sapogenin derivative separated as a voluminous mass of thin needles. It was recrystallized from 50 per cent alcohol. The mother liquor yielded material which was less pure. The yield was 6.7 gm.

This monoethyl ester is easily soluble in alcohol, acetone, chloroform, ether, acetic acid, and hot benzene. It is insoluble in ligroin. It melts on rapid heating at 257° after preliminary sintering, although samples were frequently obtained which melted at $215\text{--}218^\circ$. The sodium salt is very sparingly soluble in the presence of excess Na ions.

$[\alpha]_D^{25} = +24.5^\circ$ ($c = 0.53$ in 95% alcohol)

$\text{C}_{21}\text{H}_{30}\text{O}_6$.	Calculated.	C 71.76,	H 9.72,	OC_2H_5 8.69
$\text{C}_{21}\text{H}_{30}\text{O}_6$.	"	" 72.04,	" 9.37,	" 8.72
	Found.	(a) " 71.66, 71.91,	" 9.53, 9.54,	" 9.12
	"	(b) " 71.58,	" 9.36	

12.040 mg. of substance were dissolved in 10 cc. of alcohol and titrated against phenolphthalein with 0.1 N NaOH . Calculated for 1 equivalent, 0.232 cc.; found, 0.225 cc.

12.850 mg. of substance were titrated in the same way. Calculated, 0.248 cc.; found, 0.243 cc.

On refluxing with N NaOH no additional alkali than that required by the free carboxyl group was consumed.

This acid ester in chloroform solution gave a good yellow color with tetranitromethane. It is very stable towards acids. The ester group is very resistant to saponification. When heated in a nitrogen stream at $300\text{--}320^\circ$, approximately 1 mole of CO_2 besides H_2O was split off.

The Methyl Ethyl Ester, $\text{C}_{23}\text{H}_{32}\text{O}_6$ —The above acid ester was esterified with diazomethane in ether solution. The resulting neutral mixed ester proved difficult to crystallize. The amorphous precipitate obtained from aqueous acetone was dried to constant weight and analyzed.

$C_{22}H_{22}O_6$.	Calculated.	C 72.13, H 9.84, OC_2H_5	+ OC_2H_5	14.29
$C_{22}H_{20}O_6$.	"	" 72.40, " 9.50, " + "	"	14.34
	Found.	" 71.85, " 9.49, " + "	"	14.61

The neutral ester was not appreciably saponified on boiling with 0.1 N NaOH.

The Ethyl Ester Diacetate—0.2 gm. of the acid ester was refluxed for 2 hours with 2 cc. of acetic anhydride and 0.1 gm. of sodium acetate. After decomposition with water the precipitate was collected. The amorphous material was dissolved in alcohol and the solution boiled down to small bulk. On cooling, a small fraction of minute needles separated, which will be described below. On further concentration of the filtrate and careful dilution a second substance, the diacetate, slowly crystallized. This was collected with 50 per cent alcohol and recrystallized from dilute alcohol. It formed needles which are readily soluble in the usual solvents. For analysis it was dried at 100° and 15 mm.

$C_{22}H_{24}O_8$.	Calculated.	C 69.72, H 9.04
$C_{22}H_{22}O_8$.	"	" 69.95, " 8.73
	Found. (a)	" 69.67, " 8.52
	" (b)	" 69.51, " 8.57

The above sparingly soluble substance, which first crystallized from alcohol, was recrystallized by solution in acetone, with addition of alcohol, followed by concentration to remove the acetone. It formed needles which did not melt on heating up to 340°. It did not dissolve in alkali.

Found. (a)	C 70.91, H 8.55, OC_2H_5	7.71
" (b)	" 70.78, " 8.69	

The Di-p-Bromobenzoate of the Ethyl Ester—The acid ester was acylated in pyridine solution with *p*-bromobenzoyl chloride. The acyl derivative was separated in the usual manner. After two recrystallizations from alcohol, it formed needles which melted at 213°.

$C_{26}H_{24}O_8Br_2$.	Calculated.	C 61.07, H 6.38, Br 18.07, OC_2H_5	5.09
$C_{26}H_{22}O_8Br_2$.	"	" 61.20, " 6.17, " 18.12, "	5.10
	Found.	" 60.74, " 6.20, " 18.08, "	5.17

The Dibasic Acid, $C_{22}H_{20}O_6$ —0.3 gm. of the acid ester was refluxed for 20 hours in a mixture of 50 cc. of amyl alcohol and 2.5

gm. of KOH. After acidification the amyl alcohol was distilled off with steam. The resinous precipitate was dissolved in ether and the latter was then extracted with 5 per cent Na_2CO_3 solution. On reacidification of the latter and extraction of the flocculent precipitate with ether, the resulting solution after being dried and concentrated left a residue which was recrystallized from very dilute alcohol. The acid formed fine needles which melted at 230° after preliminary sintering.

The acid is very easily soluble in alcohol, acetone, and acetic acid, appreciably so in ether, and practically insoluble in chloroform and benzene. In dilute alcohol it is more soluble than its precursor. It gives a pronounced yellow color with tetranitromethane. The alkyl determination was negative.

$\text{C}_{23}\text{H}_{44}\text{O}_6$.	Calculated.	C 70.97, H 9.45
$\text{C}_{23}\text{H}_{44}\text{O}_6$.	" "	" 71.26, " 9.08
	Found.	" 71.27, " 8.99

10.365 mg. of substance were dissolved in 5 cc. of alcohol and titrated against phenolphthalein with 0.1 N NaOH. Calculated for 2 equivalents, 0.506 cc.; found, 0.483 cc.

Dehydrogenation of Senegenin

30 gm. of senegenin (m.p. $280\text{--}290^\circ$) were intimately mixed with 45 gm. of selenium and heated in a 500 cc. long necked flask. The latter was provided with a meter-long vertical reflux tube fitted at the end with a bent tube leading to a bulb for collection of water and more volatile reaction products. The mixture was heated in a $\text{NaNO}_2\text{--KNO}_3$ bath quickly to 340° and kept at this temperature for 48 hours. At first water was liberated and collected in the collecting bulb with some selenium and a little oil. The reaction then quieted and apparently no naphthalene hydrocarbons refluxed steadily as is usual during the dehydrogenation of other triterpenes. On the other hand, there was a gradual accumulation of an amorphous sublimate on the cooler portions of the tube. During these experiments this accumulation was not constantly melted back into the flask, so that the reaction product therefore contained more incompletely dehydrogenated or oxygen-containing tars and a smaller amount of crystalline hydrocarbons than in the case of the dehydrogenation of the companion sapogenin

described later on. After cooling, the flask and its contents were broken and glass and all were extracted in a Soxhlet apparatus with ether for 24 hours. The undissolved portion was then extracted 14 hours more with dioxane. The two extracts were separately concentrated and the residual tars in each case fractionated by distillation or sublimation as follows:

The ether extract gave 13.5 gm. of yellow residue. The latter gave 3 gm. of a greenish yellow, soft resin from 200–280° at 5 mm. (Fraction I) and 6.2 gm. of a yellow resin at 270–300° and 3 mm. (Fraction II).

The dioxane extract gave 3.1 gm. of a green residue. Sublimation of the latter for 3 hours at 260–280° and 3 mm. gave 0.3 gm. of a white sublimate (Fraction III). Finally, on sublimation for 10 hours at 260–280° and 3 mm., 0.2 gm. more of a yellow sublimate was collected (Fraction IV). Longer sublimation gave but a trace of poorly defined material. Fraction I on refractionation was separated into two further fractions. The first was a viscous oil which boiled at 220–240° and 5 mm. No picrate could be obtained from it and all attempts to isolate Diels' hydrocarbon from it were fruitless. It possibly contained mostly incompletely dehydrogenated material. The higher fraction was collected at 260–280° and 5 mm. as a yellow tar resembling Fraction II.

Fraction II (1 gm.) was dissolved in ethyl acetate and treated with petroleum ether. After standing overnight at 0° the crystalline precipitate was collected, dissolved in benzene, and the latter was extracted with small amounts of H_2SO_4 until it no longer became colored. After washing with water, any precipitated material was redissolved by addition of ether and the extract was dried with sodium sulfate. The residue left on concentration was successively recrystallized from toluene, acetic anhydride, and butyl acetate and then sublimed at 220° and 0.2 mm. (The fraction subliming towards the end contained some higher melting dehydrogenation product and was therefore kept separate.) The substance (A) now formed lustrous leaflets which melted at 246.5° after preliminary softening. When mixed with chrysene with a melting point of 250°, a depression of 10° was noted.

In order to determine whether this hydrocarbon was the only crystalline component of this fraction, the first acetic ester mother liquors were concentrated and the residue was treated with petro-

leum ether and filtered. The amorphous substance was put through the same procedure described above. After repeated recrystallization the melting point of the substance (B) again remained constant at 246.5° and gave no depression with substance (A).

$C_{32}H_{42}$.	Calculated.	C 92.56,	H 7.44,	mol. wt. 298.17
$C_{32}H_{40}$.	"	" 92.91,	" 7.09,	" " 284.16
	Found. (A)	" 92.58,	" 7.30	
	" (B)	" 92.40, 92.43,	" 7.36, 7.44	

1.705 mg. of substance in 17.265 mg. of camphor gave a depression of 12.6°. Mol. wt. found, 282.

1.257 mg. of substance in 24.855 mg. of camphor gave a depression of 6.7°. Mol. wt. found, 272.

This hydrocarbon fluoresced a blue-violet in ultraviolet light. It resembled closely chrysene in its properties, which suggests that it may be a chrysene homologue. The crystalline forms appeared to be the same, and both substances exhibited a blue fluorescence in camphor solution. The solubilities were also indistinguishable. Finally, the ultraviolet absorption spectrum resembles that of chrysene (*cf.* Fig. 1). The maxima are, however, definitely displaced and at 3800 λ there occurs a band which is not present in the chrysene spectrum. This may be due either to an additional double bond or to a mixture with some trimethylpicene described below.

Like chrysene, the new hydrocarbon gave an unstable picrate. In attempts to prepare a dibromide, nitro derivative and quinone crystalline substances could not be isolated. The red oxidation product gave with concentrated H_2SO_4 an intensive blue color like chrysoquinone, but with a tinge, however, in the green.

This hydrocarbon was indistinguishable in appearance and properties from the hydrocarbon with a melting point of 245° obtained by Ruzicka and coworkers from hederagenin and which was kindly placed at our disposal by Professor Ruzicka for comparison. No melting point depression could be detected when the two were mixed.

Fraction III was extracted with boiling ether and the extract was decanted. The residue was dissolved in 1 liter of benzene and the solution was shaken with concentrated H_2SO_4 until the latter no longer became colored. After washing with water, any precipitated substance was redissolved by addition of sufficient

acetic ether and the solution was dried with Na_2SO_4 . The residue which remained after concentration was recrystallized in succession from butyl acetate and xylene. It was then extracted with hot acetic anhydride and then sublimed twice at 230° and 0.2 mm. The substance (C) now melted constantly at 298° .

Fraction IV was purified in the same manner as Fraction III and the resulting substance (D) melted at 297.5° . The two substances (C and D) showed no depression when mixed.

$\text{C}_{22}\text{H}_{30}$. Calculated. C 93.80, H 6.20, mol. wt. 320.15
Found. (C) " 93.84, " 6.04
" (D) " 93.70, " 6.06

1.005 mg. of substance in 25.347 mg. of camphor gave a depression of 4.7° . Mol. wt. found, 304.

The hydrocarbon (C) was compared with trimethylpicene (m.p. 297°) from oleanolic acid which was kindly placed at our disposal by Professor Ruzicka. No depression was obtained in a mixed melting point determination. The appearance and solubilities of the substances seemed to be identical, as well as the intense blue fluorescence shown in ultraviolet light. Finally, the ultraviolet absorption curves of the two substances in chloroform solution were identical (Fig. 1).

Dehydrogenation of the Companion Sapogenin, $\text{C}_{21}\text{H}_{30}\text{O}_6$

14 gm. of the sapogenin acid ester were dehydrogenated with 21 gm. of selenium at a bath temperature of $320\text{--}330^\circ$ for 48 hours. The reaction proceeded smoothly, since no appreciable liquid products formed. A crystalline substance sublimed constantly on the cooler exposed walls of the flask and were now and then melted back into the reaction mixture. The cooled mass was broken and glass and all were placed in a Soxhlet apparatus and extracted first with ether for 12 hours and then with dioxane once for 5 hours and a second time for 40 hours. The residues obtained on concentration of each of these extracts were then fractionated as given below. The dioxane residues, however, were first washed with ether or water to remove persistent amounts of solvent.

The ether extract residue (5 gm.) was a soft tar which was fractionally sublimed and divided into a number of fractions. The major portion, 2 gm., was collected as a light colored sublimate at $230\text{--}240^\circ$ and 3 mm. This material was dissolved in a small

volume of hot xylene and placed in the refrigerator. The material which separated was collected and then shaken up in 1 liter of warm benzene. The solution was filtered from a small portion which remained undissolved (40 mg.). The clear benzene solution was repeatedly extracted with concentrated sulfuric acid and then after washing with water was concentrated to dryness. The residue was recrystallized first from acetic anhydride and then sublimed at 210° and 0.2 mm. The sublimate was recrystallized from butyl acetate. 0.43 gm. of lustrous leaflets was obtained, which melted at 246–247.5°. This gave no depression with the hydrocarbon (m.p. 246.5°) from senegenin. Found, C 92.73, H 7.44. The succeeding fraction which sublimed after the previous fraction at 3 mm. and 240–250° weighed 0.51 gm. and contained somewhat more contaminated material. It was not investigated in detail.

The residue from the first dioxane extract was crystalline. It was very slowly sublimed and separated into fractions each of which was recrystallized separately from xylene. At 230° and 3 mm. 0.52 gm. of lustrous platelets was obtained, which melted at 240° after recrystallization. At 240° and 3 mm. 0.65 gm. of leaflets was collected, which melted at 242° after recrystallization. Finally, at 250–260° and 3 mm. 0.1 gm. of gray powder deposited.

The second dioxane extract contained appreciable amounts of the higher melting hydrocarbon and was amorphous. On sublimation, followed by recrystallization from xylene, the following fractions were obtained.

One at 200–220° and 3 mm. which weighed 0.6 gm. and melted at 240–245° after preliminary softening. It has not been investigated further.

The higher fraction was collected at 220–240° at 3 mm. and amounted to only 0.1 gm. This material was dissolved in 1 liter of benzene and was freed from impurities by extraction with H₂SO₄. Ether was then added and the solution was washed with water and concentrated. The residue was recrystallized successively from xylene and acetic anhydride, giving 6 mg. of substance which were then sublimed at 230° and 0.2 mm. After a final recrystallization from butyl acetate a small amount was recovered which melted at 297.5° and gave no depression with the hydrocarbon (trimethylpicene) from senegenin.

THE ABSORBABILITY OF STEROLS WITH PARTICULAR REFERENCE TO OSTREASTEROL*

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It has been shown previously (1) that the absorption of sterols from the intestinal tract is highly specific. While cholesterol is readily absorbed, other sterols differing slightly in chemical structure, particularly the phytosterols, are practically non-absorbable.

Recently one of us isolated from the oyster (2) a doubly unsaturated sterol, ostreasterol ($C_{29}H_{48}O$), which, although of animal origin, is closely related to the phytosterols (3). On catalytic hydrogenation it yields a substance which is identical with sitosterol. The question arises, is ostreasterol, which appears to occupy a position intermediate between the zoosterols and phytosterols, absorbable by the mammalian organism? In this communication we present evidence indicating that it is absorbed by mice but to a much smaller extent than is cholesterol.

EXPERIMENTAL

Mice were maintained for 10 days on a diet consisting of ground, dry rye bread to which was added a mixture of 15 gm. of Crisco, 1 gm. of sterol, and 1 gm. of cholic acid, in the proportion of 6.0 gm. (Experiment 1) or 8.5 gm. (Experiment 2) of the mixture to 100 gm. of bread. In the control series no sterol was incorporated with the fat mixture.

At the end of the feeding period the mice were killed by a blow

* This investigation was made possible by the aid of the Josiah Macy, Jr., Foundation.

on the head and the livers were analyzed for total and free cholesterol by an application (to be described in a subsequent publication) of the method of Schoenheimer and Sperry (4).

DISCUSSION

Schoenheimer and his collaborators (1, 5-8) studied the absorbability of sterols in several ways: by determining the sterol content of the whole animal, of the liver, of the blood serum, of the portal blood, and of the thoracic chyle in several species after sterol feeding; by balance experiments; and by other experimental procedures. In the present study we were restricted by the paucity of ostreasterol to the use of a procedure (6) based

TABLE I
Cholesterol in Liver after Feeding Sterols (Experiment 1)

The results are average values with standard deviations.*

Sterol added to diet	No. of mice	Cholesterol in liver		
		Total	Free	Combined
		per cent	per cent	per cent
None (controls) ..	8	0 605 \pm 0 151	0 311 \pm 0 023	0 294 \pm 0 142
Cholesterol	9	4 268 \pm 0 789	0.470 \pm 0 074	3 798 \pm 0 861
Ostreasterol	9	1 057† \pm 0 319	0 287 \pm 0 029	0 770 \pm 0 316
Sitosterol ..	10	0 402 \pm 0 080	0 258 \pm 0 030	0 144 \pm 0 062

* Calculated by the formula $\sigma = \sqrt{\sum d^2 / (n - 1)}$.

† See foot-note 1.

on the observation (9, 10) that the absorption of cholesterol is greatly increased by the feeding of bile acids. The finding is confirmed in this investigation (Table I); the livers of cholesterol-fed mice contained very large amounts of total cholesterol, far more than did the livers of control mice which received no sterol aside from that (all of plant origin) contained in the basal ration.

The livers of mice fed ostreasterol also contained more sterol (expressed as cholesterol)¹ on the average than did the livers of

¹ Ostreasterol gives less color than cholesterol with the method of Schoenheimer and Sperry. Two determinations on an acetic acid solution of ostreasterol gave 74.2 and 73.6 per cent recovery in terms of cholesterol. There also appears to be a small loss in precipitation with digitonin. Two samples were dissolved in absolute alcohol-acetone, precipitated, and determined according to the free cholesterol procedure. Recoveries in terms of cholesterol were 69.7 and 69.4 per cent.

controls. The difference is not great but statistical analysis³ (Table II) shows that it is significant. If correction were made for the low values (in terms of cholesterol) given by ostreasterol,¹ the difference would be still larger; it appears quite certain that ostreasterol is absorbed by the mouse but to a much smaller degree than is cholesterol.

As a control of the technique sitosterol was fed to one group of mice. In confirmation of previous findings with the same procedure there was no increase in the sterol content of the liver. Indeed the average value for total cholesterol was significantly lower (Table II) than that found in the controls. No satisfactory explanation for the latter result is at hand. Hummel (6) pointed out that some increase in the cholesterol content of the

TABLE II
Values of t for Comparisons of Averages Given in Table I

Comparison	t for cholesterol fractions			t necessary to establish $P = 0.01$
	Total	Free	Combined	
Controls-cholesterol	12 877	5.792	11.340	2.947
Controls-ostreasterol	3.648	1.878	3.915	2.947
Controls-sitosterol	3.667	4 169	3 000	2.921
Cholesterol-ostreasterol	11 317	6 870	9.908	2.921
Cholesterol-sitosterol	15 453	8.312	13.424	2.898
Ostreasterol-sitosterol	6.287	2 139	6 143	2.898

liver follows the feeding of bile acid without the addition of cholesterol to the diet. Our results confirm this observation to the extent of showing quite high values for total cholesterol (maximum 0.843 per cent) in some of our control mice. In the sitosterol-fed mice the direct effect of bile acid is not so evident; the highest value for total cholesterol was 0.522 per cent.

Because of this result another experiment was carried out with a larger number of mice. The procedure was identical with that used in Experiment 1 except that a somewhat greater proportion

³ The probability that random sampling is responsible for a difference between means is usually estimated from the ratio of the difference to its probable error. With small numbers of observations a more accurate estimate of the probability (P) is obtained by calculation of the value designated as t by Fisher (11). A probability of 0.01 (one chance in 100 that random sampling is responsible for the difference) was selected as the criterion of significance in the present study.

of the fat mixture (and therefore a larger proportion of cholic acid and sterol) was fed. Ostreasterol was not included, as not enough was available, but stigmasterol was fed to one group of mice.

Despite the larger ingestion of cholic acid the findings (Table III) agree quite closely with those of the first experiment. The average amount of cholesterol in the livers of the control mice was almost the same, while the sterol content of the livers of the sitosterol-fed mice again was considerably lower than that of the controls. The difference was smaller, however, and not quite significant (3.12 times its probable error). The consistent result of the two experiments suggests, nevertheless, that sitosterol feeding tends to lessen the deposition of cholesterol in the liver under

TABLE III

Cholesterol in Liver after Feeding Sterols (Experiment 2)

The results are average values with standard deviations.

Sterol added to diet	No. of mice	Cholesterol in liver		
		Total	Free	Combined
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
None (controls)...	19	0.593 \pm 0.205	0.298 \pm 0.046	0.295 \pm 0.179
Sitosterol.....	22	0.468 \pm 0.171	0.278 \pm 0.031	0.190 \pm 0.158
Stigmasterol.....	9	0.519 \pm 0.160	0.282 \pm 0.017	0.237 \pm 0.166

the influence of cholic acid, perhaps by interfering with the reabsorption of cholesterol secreted into the intestinal tract.

None of the other differences between averages in Table III approaches significance. The result shows, in confirmation of previous work (1), that stigmasterol is, like sitosterol, not absorbed by mice.

The values obtained in Experiment 2 on mice receiving no additional sterol cannot strictly be used as controls for the ostreasterol data, since the amount of cholic acid ingested was different. Nevertheless, the finding of almost exactly the same average total cholesterol percentage in the two groups of controls indicates that cholic acid exerted a maximal effect in the first experiment and so strengthens the conclusion that ostreasterol is absorbed to some extent. The difference between the average

total "cholesterol" percentage in the ostreasterol-fed mice and the corresponding value in the controls of Experiment 2 is highly significant ($t = 4.663$; necessary for $P = 0.01$, 2.779).

Free cholesterol was not determined in the experiments of Schoenheimer and his colleagues. Our results show little variability of this fraction among the animals in each of the groups (except in those fed cholesterol) and a tendency for the free cholesterol to vary with the total cholesterol (except in the ostreasterol-fed group). The latter effect is most evident in the cholesterol-fed mice in which the average free cholesterol content of the liver is significantly higher than in any of the other groups (Experiment 1). The result is in accord with a similar finding on rats reported by Sperry and Stoyanoff (12). In both experiments free cholesterol was lowest in sitosterol-fed mice, significantly so in Experiment 1. In ostreasterol-fed mice free cholesterol was slightly but not significantly lower than it was in the controls.

SUMMARY

A small but significant increase over the control level in the sterol (expressed as cholesterol) content of the liver followed the feeding of ostreasterol to mice. In confirmation of previous work a much larger deposition of cholesterol occurred after feeding cholesterol under comparable experimental conditions. The result, which indicates that ostreasterol occupies a position intermediate between the phytosterols, which are non-absorbable, and cholesterol, which is readily absorbed, is of considerable interest, since ostreasterol, though of animal origin, is closely related chemically to sitosterol.

The non-absorbability of phytosterols (sitosterol and stigmasterol) is confirmed.

We are indebted to Dr. Rudolf Schoenheimer for suggesting this investigation and for his helpful advice throughout the course of the work.

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A SYNTHESIS OF CONJUGATED BILE ACIDS

III. SODIUM TAUROCHOLATE AND SODIUM TAURODES- OXYCHOLATE

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The new synthesis for conjugated bile acids reported from this laboratory (1, 2) has been applied to the preparation of sodium taurocholate and sodium taurodesoxycholate.

These important biological substances are extremely difficult to obtain from natural sources. Almost all of the commercial samples of so called sodium taurocholate are badly decomposed, vile smelling mixtures of several bile acids, and natural sodium taurodesoxycholate has been obtained only in an impure amorphous state by Gullbring (3).

The latter substance has been synthesized by Wieland (4) by employing the Bondi and Mueller method (5), which in our hands yielded the sodium salt and not the free taurodesoxycholic acid. The substance isolated was unquestionably the sodium salt and not the free acid as reported.

Triformylcholic Acid and Diformyl-desoxycholic Acid—In the preparation of these substances (1, 2) it is best to filter at once as soon as crystallization is complete, to avoid low yields.

Sodium Taurocholate—The fresh acid chloride (2) from 25 gm. of triformylcholic acid (m.p. 210° corrected) is added to a solution of 53 gm. of NaOH (95 per cent) and 250 gm. of taurine in 900 cc. of water at room temperature. The mixture is well shaken for about 5 hours. The solution should now be clear and only faintly alkaline to phenolphthalein paper, if at all. If marked opalescence is present and a large amount of taurine has precipitated, not enough sodium hydroxide has been employed (see "Discussion"). The solution is neutralized to litmus at 0° with concentrated HCl

(about 80 cc.). 2 liters of ice-cold acetone are stirred in and the recovered taurine is filtered after 5 minutes, and washed with more acetone. The recovery here amounts to 205 to 220 gm.

No alkali should be added to the filtrate at this point, but a few drops of acid are added if necessary to restore neutrality. The large volume of solution may be spontaneously evaporated in a dish for 2 days. It is then concentrated under reduced pressure in a bath at 40–50°, with the aid of a few drops of capryl alcohol, until a heavy oil separates out (375 to 650 cc.; the most successful runs will be nearer the larger figure). The contents of the flask are transferred to a large dish and stored in a refrigerator overnight. The supernatant liquid is concentrated again to about 150 cc. and any additional gum obtained here is added to the main lot to be thoroughly dried over fresh anhydrous calcium chloride in a vacuum desiccator. (The pump should be run continuously.)

The pulverized residue is extracted with a total of 450 cc. of boiling 95 per cent alcohol, and ether added cautiously to a volume of 1 liter. After storing in the ice box overnight, the precipitate is filtered by suction, washed well with ether, and thoroughly dried in a vacuum desiccator. The yield is 21 to 22 gm. and consists of sodium triformyltaurocholate.

If the yield is markedly lower, the filtrate is evaporated to dryness and a 10 per cent solution made of the dried residue in 95 per cent alcohol. After the addition of 1 cc. of water to every 40 cc. of solution, ether is added to 6 times the volume. This final fraction of sodium triformyltaurocholate is added to the main lot.

The crude product is dissolved in 7 times its weight of water and to this solution is added one-half its volume of 12 per cent sodium hydroxide. After cooling for $\frac{1}{2}$ hour, the solution is neutralized to litmus with concentrated hydrochloric acid. It is now shaken with one-third its weight of sodium chloride for 2 minutes, saturated with ether, and stored in the ice box overnight. The crystalline precipitate is filtered by gentle suction on a large Buchner funnel, washed with a little *ice-cold* saturated sodium chloride solution saturated with ether, dried to constant weight over fresh anhydrous calcium chloride in a vacuum desiccator, and extracted with a total of 200 cc. of boiling commercial absolute alcohol. If the trace of salt present in colloidal solution fails to precipitate within 1 hour, it is brought down by the addition of 10 cc. of water.

After 24 hours the salt is filtered with the aid of a minimal amount of charcoal. The solution is finally diluted with 10 cc. more (20 cc. in all) of water and sufficient ether to incipient cloudiness, which is dissipated by the addition of a few drops of alcohol. The optimum water concentration is 9 to 12 per cent, before the addition of ether. After 24 hours the crystals are thoroughly washed on the Buchner funnel with a mixture of ether and 95 per cent alcohol (7:3) and air-dried. The yield from a successful run is 15 gm. If it is less, the mother liquor is evaporated to dryness, the residue taken up in water, and the sodium taurocholate salted-out again with sodium chloride and ether.

The remainder of the taurine is obtained by adding 600 cc. of 95 per cent alcohol to the 150 cc. of residual liquor, strongly acidifying with concentrated hydrochloric acid, and storing in the ice box. The dry precipitate is extracted with concentrated hydrochloric acid, the extract boiled down one-half, diluted once with water, and treated with charcoal. The filtrate is boiled down until crystals appear and the precipitation completed with 5 volumes of alcohol.

Sodium taurocholate crystallizes in sheaves of long hair-like needles, frequently compounded to appear as rosettes.

$C_{16}H_{44}O_7NSNa$.	Calculated.	C 58.21, H 8.02, S 5.97, Na 4.29
537	Found.	" 58.40, " 8.42, " 6.04, " 4.05

A separate determination for moisture was made *at the same time* that the samples for the analyses were weighed and the appropriate correction subsequently made.

Sodium taurocholate, dried to constant weight over P_2O_5 at 138° and at 10 mm. pressure, has the following specific rotation.

$$[\alpha]_D^{25} = \frac{+0.87^\circ \times 100}{1 \times 3.680} = +23.7^\circ \text{ (in water)}$$

The decomposition points for the normal form of sodium taurocholate, determined by immersion in preheated baths, are not easily reproducible. The same values (130 – 145°) were obtained with our material, a sample of the natural salt, and a sample made according to a modification of the Bondi and Mueller method.¹

¹ A practical procedure for the Bondi and Mueller synthesis will soon be published.

The para form decomposes² over a range of 225–235°, without discoloration.

The amount of water present in the pure sodium salts of the bile acids, both conjugated and unconjugated, depends entirely upon the prevailing relative atmospheric humidity, and hence the substances have no definite percentage of moisture corresponding to a definite number of molecules of water of crystallization.³

Sodium Taurodesoxycholate—The coupling reaction is carried out with 42.1 gm. of NaOH (95 per cent), 175 gm. of taurine, 900 cc. of water, and the fresh acid chloride from 25 gm. of diformyl-desoxycholic acid (m.p. 193–194°). The shaking requires 24 hours. The solution, which should be clear and strongly alkaline to phenolphthalein, is worked up as described for sodium taurocholate. The gummy layer obtained in the ice box is poured into a beaker. After 1 hour the exudate is poured off and the residual material dissolved in water to make a total volume of 400 cc. The solution is heated to 60° and to it is added a solution of 17.5 gm. of NaOH in 16 cc. of water. The clear solution is transferred *at once* to a separatory funnel. A heavy oil soon begins to separate. After 24 hours at room temperature, but not longer, the lower layer is cautiously drawn off into a lipped dish.⁴

The viscous liquid is neutralized to litmus at 0° with concentrated hydrochloric acid, and placed over fresh anhydrous calcium chloride in a vacuum desiccator. (There is danger of foaming for the first half hour. The pump should run continuously.) After 24 hours the hard gum is taken up in 200 cc. of boiling 95 per cent alcohol. The filtrate, seeded with sodium taurodesoxycholate, is allowed to stand for 24 hours at room temperature. Ether is finally added every 24 hours, each time to incipient cloudiness, to a total volume of 500 cc. The sodium taurodesoxycholate, in rosettes of hard prismatic needles, is filtered by suction, washed

² The solid normal form is converted to the solid para form on slow heating.

³ The amount of water bound may vary from 2 to 250 per cent, depending upon the substance and conditions. Impure substances are unusually hygroscopic.

⁴ The procedure is necessary not to deformylate but to salt-out a minute amount of contaminating substance. It is analogous to the salting-out of sodium taurocholate with NaCl and ether, without which crystallization is practically impossible.

with a mixture of alcohol and ether (1:1), and air-dried. The yield is 18 to 21 gm.

The samples for analysis were prepared as described for sodium taurocholate.

$C_{26}H_{44}O_6NSNa$.	Calculated.	C 60.00, H 8.27, S 6.15, Na 4.42
521	Found.	" 59.56, " 8.72, " 6.09, " 4.30

Sodium taurodesoxycholate dried to constant weight over P_2O_5 at 138° and at 10 mm. pressure has the following specific rotation.

$$[\alpha]_D^{25} = \frac{+1.36^\circ \times 100}{1 \times 3.840} = +35.4^\circ \text{ (in water)}$$

The normal form of sodium taurodesoxycholate swells greatly with considerable foaming when immersed in a bath preheated to 117° .

The para form decomposed between 160 – 175° with no discoloration. The same remarks apply here as in the case of para-sodium taurocholate.

DISCUSSION

The poorer the quality of formylated acids available, the less sodium hydroxide is needed for the coupling action. The purity of the thionyl chloride is also a factor. It is therefore advisable to make a sufficiently large amount of formylated acid and to distil a corresponding quantity of thionyl chloride beforehand. The formylated acid is then standardized, so to speak, by making experimental 1 gm. runs. The amounts of solid sodium hydroxide suggested for these runs are 1.6, 1.8, 2.0, and 2.2 gm.; the yields obtained are compared as to weight, color, hygroscopicity in air, and crystalline appearance under the microscope. The crystals in rosettes of short, hard prismatic needles from 86 per cent alcohol and ether should not be gummy. If the large run is then not quite satisfactory, it is suggested that 5 gm. more of NaOH be used in the next one.

When the conditions for crystallization are ideal, pure glycolic and glycodesoxycholic acids and their sodium salts, and pure sodium taurocholate and sodium taurodesoxycholate will all start to crystallize out within 1 minute.

The total amount of taurine recovered is about 92 per cent of the amount used.⁵

We have found that ferric chloride fails to precipitate sodium taurodesoxycholate in the presence of taurocholate. On the other hand, Tengström (7) showed that sodium taurocholate can be precipitated by saturation with NaCl with a recovery of 88 per cent. He pointed out that the procedure is useful only when the amount of other contaminating substances present is at a minimum.

The recent paper of Tanaka (8) on sodium taurocholate contains numerous errors and misstatements. This can easily be appreciated by careful study of Hammarsten's paper (9) and the work reported here. The normal form of sodium taurocholate does not change to the para form in aqueous solution.

We wish to express our sincere thanks to Dr. Louis Bauman for his many friendly suggestions. The authors also wish to thank Mr. W. Saschek for the microanalyses.

SUMMARY

1. Practical details have been given for new syntheses of sodium taurocholate and sodium taurodesoxycholate in yields of 50 and 70 per cent respectively.

2. There is no difference between the properties of natural and synthetic sodium taurocholate.

3. The decomposition points of the normal and para forms of sodium taurocholate and sodium taurodesoxycholate are not useful, except in a general way, for characterizing them, since they decompose over a wide range and the values obtained depend upon the rate of heating and other conditions.

4. The sodium salts of the conjugated and unconjugated bile acids do not crystallize with a fixed percentage of water corresponding to a definite number of molecules of water of crystallization. The amount of bound water varies according to the prevailing relative humidity of the atmosphere to which they happen to be exposed.

⁵ The large amounts of taurine necessary may be conveniently and inexpensively prepared by the procedure recently published (6).

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THE EFFECT OF ACID HYDROLYSIS ON THE YIELD OF ANDROGENIC AND ESTROGENIC ACTIVITIES FROM HUMAN URINE*

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(Received for publication, March 10, 1937)

In a previous paper Gallagher, Koch, and Dorfman (1) confirmed on normal human urine the findings of Marrian (2), Borchardt, Dingemanse, and Laqueur (3), Cohen and Marrian (4), Zondek (5), and others that acid hydrolysis of pregnancy urine increases the yield of estrogenic activity extractable by immiscible solvents. As a result of the work of Cohen and Marrian (6) this is interpreted to mean that a conjugated estrogenic form is hydrolyzed by the acid treatment. Inasmuch as the yield of androgenic activity was not markedly altered by boiling the urine for 2 or more hours with acid as compared with no hydrolysis, the conclusion seemed warranted that androgenic substances are not present in conjugated form in urine and also that the urinary androgens are not acid-labile. More recently we have shown that these conclusions are not correct for we now find that brief acid hydrolysis increases the yield of androgens very strikingly, but also that longer hydrolysis destroys an appreciable amount of the activity.

EXPERIMENTAL

A composite stock of urine from normal young men was acidified with one-tenth its volume of commercial hydrochloric acid and the samples boiled for 0, $\frac{1}{2}$, 1, 2, 6, and 12 hours respectively before they were extracted in the Gallagher and Koch (1) extractor. The separation and assays of the androgenic and estrogenic activities were carried out by the methods described elsewhere (7).

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The results are given in Fig. 1 and Table I. Curve B represents the samples above. Curves A and C give the results on two other

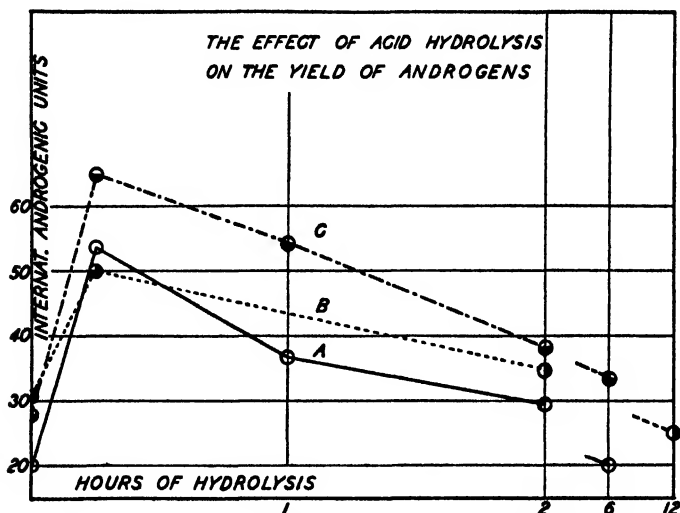


FIG. 1. Curves A, B, and C show the yields of international androgenic units obtained on three different samples of urine with 10 per cent by volume commercial hydrochloric acid for the time indicated.

TABLE I

Effect of Acid Hydrolysis on Yield of Estrogenic Activity from Urines A and B

Time boiled	Estrogenic activity expressed as theelin per liter urine	
	Urine A	Urine B
hrs.	micrograms	micrograms
0	0	0
$\frac{1}{2}$	18	9
1	13	No extraction
2	20	11
6	5	No extraction
12	No extraction	11

collections of urine from men, which were allowed to stand for 1 to 2 weeks at room temperature after the addition of 1 to 2 per cent hydrochloric acid. Before they were extracted the content

of acid was increased to 10 per cent hydrochloric acid by volume and then treated in the same way as Urine B.

The results show that the 15 minute acid hydrolysis at boiling temperature increased the yield of androgenic activity 70 to 160 per cent over the no boiling procedure and 35 to 78 per cent over the 2 hour treatment. The liberation and subsequent loss are obvious. That the estrogenic material in the urine of normal men is completely liberated by the 15 minute boiling with 10 per cent hydrochloric acid by volume and that there is no destruction is obvious from Table I. It remains to be determined whether this is true for pregnancy urine.

Urine from normal women behaves in the same way in our hands thus far. We find an average increase of 66 per cent in androgenic activity by the 15 minute boiling procedure as compared with the 2 hour treatment. In fact, urine from an apparently normal woman (for details see Fig. 2 in the paper by Gallagher, Peterson, Dorfman, Kenyon, and Koch (7)) gave an average of 56 international androgenic units per 24 hours after 15 minutes hydrolysis, whereas urine from three other normal women, by the 2 hour boiling treatment, gave 25, 28, and 25 international androgenic units respectively per 24 hours.

DISCUSSION

Our tentative conclusions are that one or more androgenic substances are present in human urine in part in a conjugated or combined form which is not extractable in an active form by benzene, but that one or more of these forms are very acid-labile, while another is acid-resistant. It remains to be determined whether the loss can be ascribed to the formation of chlorodehydroandrosterone as first observed by Butenandt and Dannenbaum (8), whether androgenic substances may, like cholesterol, be altered by strong hydrochloric acid at boiling, or whether still other androgenic substances are involved. We have determined that it is not an oxidative destruction, because boiling in an atmosphere of carbon dioxide yields the same results as in air. Preliminary quantitative studies indicate that androsterone is not destroyed by acid hydrolysis. Further studies are under way to determine the factors involved in the hydrolytic increase and destruction of androgenic activity.

SUMMARY

Androgenic activity can be extracted from acidified unboiled urine by benzene. Acid hydrolysis for 15 minutes liberates 70 to 160 per cent more androgenic activity easily extracted by benzene. Acid hydrolysis for 2 or more hours again lowers the yield of androgenic activity to approximately 40 per cent less than after 15 minutes hydrolysis. The importance of these findings in quantitative studies on the urinary excretion of androgenic substances is obvious.

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NOTE ON THE CONFIGURATIONAL RELATIONSHIP OF ALKYL HALIDES AND 2-HALOGENO ACIDS

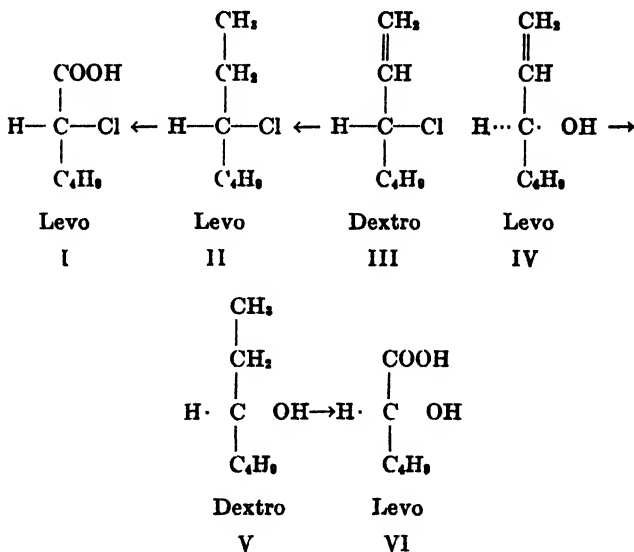
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(Received for publication, March 19, 1937)

In a series of publications from this laboratory the correlation between the configurations of secondary alkyl halides of type (II) and of halogeno acids of type (I) was formulated as in formulæ (I) and (II).



This formulation was based on the assumption that configurationally related saturated and unsaturated alkyl halides (II) and (III) differ in the sign of their rotations in a manner similar to the

change in rotation observed on passing from the unsaturated carbinol (IV) to the saturated carbinol (V).

Many earlier attempts to substantiate this assumption by direct experiment were unsuccessful. The product of hydrogenation of the unsaturated halide was always a hydrocarbon. It has now been found that it is possible to convert the unsaturated halide into the saturated halide by carrying out hydrogenation in solution in methyl alcohol containing hydrogen chloride.

Hydrogenation of substance (III) yields a substance (II) of opposite sign of rotation, similar to the results obtained for the hydrogenation of substance (IV). Thus the earlier formulation is now substantiated by direct experiment.

TABLE I

Direction of Rotation of Configurationally Related Substituted Normal Alkyl and Normal α -Substituted Carboxylic Acids

$R_1 < R_2$		$R_1 < R_2$		$R_1 < R_2$	
$\begin{array}{c} R_1 \\ \\ H - C \\ \\ R_2 \end{array}$	$\begin{array}{c} COOH \\ \\ H - C \\ \\ R_2 \end{array}$	$\begin{array}{c} R_1 \\ \\ H - C \\ \\ R_2 \end{array}$	$\begin{array}{c} COOH \\ \\ H - C \\ \\ R_2 \end{array}$	$\begin{array}{c} R_1 \\ \\ H - C \\ \\ R_2 \end{array}$	$\begin{array}{c} COOH \\ \\ H - C \\ \\ R_2 \end{array}$
$OH \rightarrow$	OH	$NH_2 \rightarrow$	NH_2	$H - C - Cl \rightarrow$	$H - C - Cl$
Dextro	Levo	Dextro	Levo	Dextro	Dextro

R_1 and R_2 stand for alkyl groups.

It may be added that the partial rotation of the chlorine atom has the same sign in each of the three chlorides. The difference in the direction of rotation of substance (III) and substances (II) and (I) is due to the fact that in the visible region the rotation of the latter two substances is determined by the partial rotation of the chlorine atom, whereas the partial rotation of the ethylenic group determines the rotation of substance (III).

Dispersion measurements of substance (III), 3-chloro-1-heptene (vinylbutylchloromethane), lead to the formula

$$[M]_{\text{least max}}^{\text{ss}} = \frac{49.5878}{\lambda^2 - 0.0365} - \frac{45.4628}{\lambda^2 - 0.029}$$

The constant $\lambda_1^2 = 0.0365$ corresponds to the first absorption band of the ethylenic group.

Thus, on the basis of the work done in this laboratory, the configurational relationships are now established between secondary carbinols and α -hydroxy acids,¹ between amines and α -amino acids,² and between secondary halides and α -halogeno acids.³ The relationships are given in Table I.

EXPERIMENTAL

Levo-3-Chloro-1-Heptene (Vinylbutylchloromethane)—20 gm. of 1-heptene-3-ol (vinylbutylcarbinol), $[\alpha]_D^{25} = +10.5^\circ$ (homogeneous), which was prepared and resolved as described by Levene and Haller,⁴ were treated with phosphorus pentachloride by the usual method.⁵ The chloride distilled at $87-90^\circ$ ($p = 92$ mm.). Yield 13 gm.

$$[\alpha]_D^{25} = \frac{-3.50^\circ}{1 \times 0.886} = -3.95^\circ; \quad [M]_D^{25} = -5.24^\circ \text{ (homogeneous)}$$

Dextro-3-Chloroheptane—13 gm. of 3-chloro-1-heptene, $[\alpha]_D^{25} = -3.95^\circ$ (homogeneous), were dissolved in 25 cc. of methanol, and 10 cc. of a saturated solution of hydrogen chloride in methanol were added. 0.3 gm. of Adams' catalyst was added, and the mixture was shaken in hydrogen at an initial pressure of 45 pounds per sq. inch for 16 hours. The final pressure was 30 pounds per sq. inch (2 atmospheres).⁶ The catalyst was filtered off and the filtrate was poured into concentrated aqueous calcium chloride solution and extracted with pentane. The extract was washed with concentrated calcium chloride solution, water, dilute potassium carbonate, and water. It was finally dried over phosphoric anhydride overnight. On distillation, a hydrocarbon fraction was obtained which boiled at 92° ($p = 1$ atmosphere). The higher fraction boiled at $60-100^\circ$ ($p = 150$ mm.). Yield 5 cc. $\alpha_D^{25} = +1.35^\circ$ (homogeneous, 2 dm.). This fraction was redistilled.

¹ Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **79**, 475 (1928).

² Levene, P. A., and Mardashew, S., *J. Biol. Chem.*, **117**, 707 (1937).

³ Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **81**, 425, 703 (1929); **83**, 177, 185, 579, 591 (1929).

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⁵ Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **81**, 425 (1929).

⁶ Burgess Parr Company, East Moline, Illinois, model apparatus.

After a forerun was taken, a fraction distilled at 87–90° ($p = 113$ mm.). Yield about 1 gm., having $n_D^{25} = 1.4221$; $\alpha_D^{25} = +1.46^\circ$ (homogeneous, 1 dm.).

3.602 mg. substance: 8.280 mg. CO_2 and 3.720 mg. H_2O

6.470 " " : 6.895 " AgCl

$\text{C}_7\text{H}_{11}\text{Cl}$. Calculated. C 62.42, H 11.23, Cl 26.35

134.6 Found. " 62.69, " 11.45, " 26.21

THE CARBON METABOLISM OF *GIBBERELLA SAUBINETII* ON GLUCOSE*

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(Received for publication, March 13, 1937)

Gibberella saubinetii is a fungus of the *Fusarium* genus and is often the causal agent of head and seedling blight of small grains. In the case of barley the disease produced by this fungus is known as barley scab, and its presence results in large economic losses, since often the diseased barley is heavily discounted in the grain markets. In addition, "scabby" barley is definitely toxic to animals with simple stomachs, such as the horse and pig, but apparently animals with complex stomachs, such as cattle and sheep, are not affected (13). The toxic symptoms usually involve vomiting and refusal of food. Roche and Bohstedt (13) did not isolate the toxic principle but believe it to be associated with the glucoside or basic nitrogen fractions. Raistrick and coworkers (12) have made an extensive study of the carbon balance of a large number of fungi with the object of using the carbon balance studies as a means of classifying the molds. A section of their report deals with some *Fusarium* species but does not include *Gibberella saubinetii*. Other studies of *Fusarium* species are those by Tochinal (16, 17) and Anderson (1) on *Fusarium lini* and Anderson, Everitt, and Adams (2) on *Fusarium oxysporum*. The chief metabolic products resulting from the growth of *Fusaria* on sugars appear to be carbon dioxide and ethyl alcohol. Anderson found *Fusarium lini* to grow equally well on glucose, galactose, maltose,

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lactose, levulose, xylose, inulin, and soluble starch and apparently to show no discriminating preference among these carbohydrates. In the absence of carbohydrates but with ethyl alcohol as the sole source of carbon, it metabolized the alcohol and built mycelium at a relatively rapid rate.

Studies dealing with the biochemistry of *Gibberella saubinetii* appear to be limited to studies of hydrogen ion effects (9, 10), to effects of inorganic ions (10), or to qualitative identification of the enzymes of the organism. Spitzer and Diehm (15) identified glucosidase, invertase, catalase, lipase, and proteolytic enzymes of the tryptic and ereptic type in the organism grown on malt extract.

EXPERIMENTAL

Culture and Culture Medium—The culture of *Gibberella saubinetii* used in these studies was secured from Dr. Carl Eide of the Department of Plant Pathology of the University of Minnesota, who had isolated it and studied it in pure culture (5). It had been maintained on potato-dextrose-agar slants. The inorganic medium used in our studies was that used by Tochinali (16) and Anderson (1) in their studies on *Fusarium lini* and by Anderson, Everitt, and Adams (2) in investigating *Fusarium oxysporum*. It consisted of NH_4NO_3 1.00 gm., MgSO_4 0.25 gm., KH_2PO_4 0.50 gm., and water to make 1000 cc. Pure dextrose was added to the stock solution to make an approximately 4 per cent glucose solution. The exact amount of glucose in the final solution was determined by a Shaffer and Hartmann (14) titration and the Hagedorn and Jensen (7) method as modified by Hanes (8), together with a carbon determination on an aliquot of the culture medium. The hydrogen ion concentration of the culture medium was determined electrometrically, with a Bailey (3) electrode, both immediately after sterilization and at the end of the selected growth period.

Culture Flasks and Temperature of Incubation—300 cc. of medium were placed in 500 cc. flasks and sterilized at 15 pounds pressure for 20 minutes. The flasks were arranged for daily aeration by placing glass tubes through a 2-hole stopper. The tubes were plugged with cotton filters and closed with glass plugs. The sterilized flasks were allowed to stand several days to make it possible to detect any contamination which might have escaped

sterilization. Cultures of the organism, which had been transplanted at regular intervals and grown for 3 days on agar, were then emulsified with 5 cc. of sterile water and added directly to the glucose-liquid medium. The stoppers were adjusted and sealed with paraffin, the rubber connections closed with glass plugs, and the flasks placed in an incubator maintained at 26-28°.

Determination of Carbon Dioxide—Carbon dioxide was determined at frequent intervals during the growth period in order to insure against the development of pressure within the flask. The air which was forced through the fermentation flasks was rendered CO₂-free by bubbling through 40 per cent NaOH. Attached to the fermentation flask was a sulfuric acid trap to catch any volatile organic matter which might be removed from the culture medium. The carbon dioxide was caught in a series of three barium hydroxide absorption tubes, similar to the type used by Neal and Palmer (11). After aeration, the excess barium hydroxide was titrated with standard hydrochloric acid, with phenolphthalein as an indicator. When the culture was ready for final analysis, the long tube was forced below the surface of the fermentation liquor and aeration continued for 90 minutes to insure complete removal of the last traces of CO₂ in solution.

Determination of Titratable Acidity—10 cc. of fermentation solution were brought to boiling and titrated with 0.1 N sodium hydroxide with phenolphthalein as an indicator. The titratable acidity was recorded as the number of cc. of 0.1 N base required to neutralize 10 cc. of fermentation liquor.

Determination of Dry Matter and Carbon in Mycelium—Upon the completion of the experimental period the mycelium was filtered through a weighed Gooch crucible, washed with hot water, and dried to constant weight in a hot air oven at 110°. The filtrate was made to volume and used for subsequent determinations. The carbon in the original culture medium, in the mycelium, and in the various fractions of the residual solution, was determined by chromic acid wet combustion, the carbon dioxide formed being absorbed in barium hydroxide and titrated as indicated above.

Determination of Glucose—Glucose in the residual liquors was determined by the Hagedorn and Jensen (7) method as modified by Hanes (8). An aliquot of the filtrate from the mycelium was diluted so that 1 cc. contained from 1 to 2 mg. of glucose.

Determination of Volatile Neutral Compounds—After the determination of pH, titratable acidity, and sugar, 250 cc. of fermentation solution remained from the original 300 cc. This was made alkaline to pH 8 by means of NaOH, and 100 cc. were distilled off to be analyzed for carbon content by chromic acid wet combustion. This value is recorded in Table I as carbon in volatile neutral compounds.

Determination of Non-Volatile Acids—After distilling off 100 cc. for the volatile neutral compounds, 150 cc. remained which contained the sodium salts of the organic acids in the original 250 cc. of fermentation liquor. The method of Raistrick and coworkers (12) was followed for the determination of non-volatile acids. 50 cc. of the remaining 150 cc. were placed in a 250 cc. volumetric flask and 150 cc. of 95 per cent alcohol added. The solution was neutralized to phenol red with ammonium hydroxide and 5 cc. of 20 per cent calcium acetate solution were added, after which the volume was made to 250 cc. with 95 per cent alcohol. The precipitate which formed was allowed to stand overnight and then filtered on asbestos in a weighed Gooch crucible, washed with 80 per cent ethyl alcohol, and dried at 110°. This precipitate of the calcium salts of the non-volatile organic acids was then used for a carbon determination by introducing the Gooch crucible and contents directly into the wet combustion flask.

Determination of Volatile Acids—The remaining 100 cc. from the 150 cc. left after the determination of volatile neutral compounds were used for the determination of volatile acids by the method of Fred, Peterson, and Davenport (6), in which 40 gm. of Na_3PO_4 and 25 cc. of H_3PO_4 were added to the 100 cc. of fermentation liquor. The distillation was carried out until no more acidic distillate came over. The distillate was titrated with standard $\text{Ba}(\text{OH})_2$, evaporated to dryness, taken up in carbon dioxide-free water, filtered, and made to 25 cc. volume to be used for carbon determination by chromic acid wet combustion.

Results

Fourteen flasks with 300 cc. of medium containing a known quantity of glucose as the only source of carbon were inoculated with a spore and mycelium suspension of *Gibberella saubinetii*. Two flasks were removed at intervals of 10 to 13 days and analyzed

TABLE I
Average Carbon Metabolism of *Gibberella saubinetii* on Glucose Media over a Period of 56 Days
Initial pH = 4.29. Total carbon in 300 cc. of solution, 4.2096 gm.

Days grown.....	Carbon									
	13	25	36	46	56	13	25	36	46	56
CO ₂ evolved.....	0.2333	0.6203	0.8480	1.2525	1.1472	5.54	14.73	20.86	29.77	27.25
Carbon in mycelium.....	0.0965	0.2063	0.2508	0.3097	0.2640	2.29	4.91	5.96	7.35	6.27
Total.....	0.3298	0.8266	1.0988	1.5622	1.4112	7.83	19.64	26.82	37.12	33.52
Residual solution										
Sugar.....	3.5900	2.5525	1.4748	0.6560	0.6322	85.23	60.67	35.04	15.60	15.03
Volatile neutral compounds..	0.2360	0.7566	1.1800	1.6250	1.7894	5.60	17.99	28.89	38.61	42.49
Non-volatile acids.....	0.0436	0.0893	0.1140	0.1420	0.1484	1.04	2.12	2.71	3.37	3.53
Volatile acids.....	0.0073	0.0117	0.0133	0.0098	0.0091	0.18	0.28	0.32	0.23	0.22
Carbon in H ₂ SO ₄ trap.....	0.0015	0.0020	0.0032	0.0047	0.0066	0.04	0.05	0.08	0.11	0.16
Total.....	3.8784	3.4121	2.7853	2.4375	2.5857	92.09	81.11	67.04	57.92	61.43
Grand total.....	4.2082	4.2387	3.8841	3.9997	3.9969	99.92	100.75	93.86	95.04	94.95

Titer of Solution				
0.1 N NaOH to titrate 10 cc. solution, cc.....	1.02	1.48	2.46	1.89
pH at end of fermentation....	3.13	3.32	2.49	3.13
				2.35
				2.70

for carbon dioxide, carbon in the mycelium, glucose, carbon in the volatile and non-volatile acids, carbon in the volatile neutral compounds, together with the pH and titratable acidity. Before inoculation the pH and glucose were determined. In general, the analyses from duplicate cultures agreed with each other within rather narrow limits. The average data are presented in Table I in terms of the percentage and gm. of carbon of the original glucose present in each fraction.

Qualitative Determination of Metabolic Products—A series of mass cultures of *Gibberella saubinetii* was grown in liter flasks on the glucose-inorganic salt culture medium. The fermentation liquors from these cultures were combined and distilled with a fractionating column, until a fraction was obtained which boiled between 77–79°. This fraction proved to be largely ethyl alcohol. It gave the iodoform test, and positive identification of ethyl alcohol was obtained by forming the ethyl 3,5-dinitrobenzoate, which melted at 91.5–92.5° (given in the literature, 92–93°).

Qualitative tests for aldehydes were also applied to this fraction, and indications of their presence were obtained with Schiff's reagent, Nessler's reagent, and sodium nitroprusside. Attempts to form derivatives with *p*-nitrophenylhydrazine and dimethylhydroresorcinol failed. If aldehydes were present, they were present in very small amounts.

Citric and tartaric acids were identified in the non-volatile acid fraction. The acids were isolated in the pure state by first preparing the calcium salts, separating these approximately by differential solubilities, liberating the organic acids from these salts with sulfuric acid, and extracting the free acids with ether in a wet extraction apparatus. Tartaric acid crystallized from the concentrated ether solution on standing in the cold, m.p. 169° (given in the literature, 170°). Tartaric acid was further confirmed by calcium and organic carbon analyses of the calcium salt as well as by other qualitative tests. Citric acid was extracted from the solid acid residue by hot chloroform and recrystallized from chloroform, m.p. 151–152° (given in the literature, 153°). Qualitative tests checked with those for citric acid. Oxalic acid was not present.

The volatile acids were distilled from fermentation liquor after the volatile neutral compounds had been removed by distillation.

The odor of acetic acid was readily detectable in the distillate. Dyer's test for the free fatty acids (4), with amyl alcohol and ferric chloride, indicated acetic acid to be the only volatile acid present. The calculated neutral equivalent on the distillate was 60.33, while the theoretical neutral equivalent for acetic acid is 60.03.

DISCUSSION

The principal products of the fermentation of glucose by *Gibberella saubinetii* are carbon dioxide and ethyl alcohol. Although these products are produced slowly at first, the fermentation rate rises rapidly by the end of the 13 day period, reaching a high point by the end of the 46 day period. Organic acids were produced slowly throughout the incubation period with the non-volatile acids, reaching a high point of 3.53 per cent of the total carbon at the end of the 56th day. The volatile acids remained fairly constant throughout the experiment and never exceeded 0.32 per cent of the total carbon.

The mycelium grew at the surface of the liquid in the form of a pellicle in all samples studied, except in one flask of the 56 day series, where it grew completely submerged. The high point for the carbon transformed into mycelium was 7.35 per cent of the total carbon at the end of the 46 day period.

Practically complete recovery of the original carbon is realized on samples taken off for analysis at the end of the 13 day and 25 day periods. During the remainder of the experiment, total recovery of carbon ranges between 94 and 95 per cent of the total carbon. We cannot explain our failure to recover all of the carbon in these later series.

The initial pH of the medium at the start of fermentation was 4.29. At the end of the 13 day period the pH had decreased to 3.13 where it remained fairly constant throughout the remainder of the experiment.

SUMMARY

The carbon metabolism study of *Gibberella saubinetii* was carried out on a glucose-artificial salt medium. Data reported are: carbon in the carbon dioxide, carbon in the mycelium, carbon in the volatile neutral compounds, carbon in the non-volatile acids, and carbon in the volatile acids, together with the titratable acidity and pH of the solution during fermentation.

Ethyl alcohol was identified as the almost exclusive constituent in the volatile neutral compounds fraction, although qualitative tests indicated traces of an aldehyde to be present. Tartaric and citric acids were identified in the non-volatile acid fraction. Acetic acid was the only acid present in the volatile acid fraction.

The principal metabolic products of *Gibberella saubinetii* are carbon dioxide and ethyl alcohol, which indicates that the organism causes a rather typical alcoholic fermentation. In this respect it resembles other *Fusaria* which have been studied.

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CREATINE AND CREATININE EXCRETION IN INFANCY*

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The period of infancy is peculiarly suited to a study of creatine and creatinine metabolism for several reasons. The quantity of musculature present at birth and its rate of growth relative to that of the body as a whole have been determined through anatomical studies (1). The diet of the infant is relatively simple and constant in character over a long period of time; and infancy is a period of rapid growth and development. While the literature abounds with short term studies of creatine-creatinine metabolism in infancy (2), many of the studies are difficult to interpret. Often the subjects were convalescent, rather than well infants; dietary habits of the infants previous to the study are not recorded, and conditions of study differ so much that no complete picture of creatine-creatinine metabolism during the 1st year of life yet exists.

The present report records the urinary creatine and creatinine excretions during infancy as determined from 424 3 day studies of 52 healthy infants from 2 weeks to 1 year of age, and from 73 24 hour studies of twenty-three normal infants during the 1st week of life. The distribution of the data is shown in Table I. The new born infants, with two exceptions, were fed human milk; the infants above 2 weeks of age were given curded whole milk feedings with 6 per cent of added carbohydrate. The diet after the 2nd week of life thus contained much more protein and was of higher caloric value than the diet of the new born infants. The customary vitamin supplements were given. The general health and the rates of growth and development of the infants were excellent.

* This study was aided by a grant from Mead Johnson and Company, Evansville, Indiana.

Urines were collected under toluene and each 24 hour specimen was analyzed separately. Folin's micromethod was used for creatinine (3); creatine was converted to creatinine by autoclaving

TABLE I
Distribution of Data of Creatine and Creatinine Excretion of Infants

Age range	No. of infants	No. of periods*	Average weight	Age range	No. of infants	No. of periods*	Average weight
<i>days</i>			<i>kg.</i>	<i>wks.</i>			<i>kg.</i>
1-2	23	41	3.4	20-25	37	79	7.2
3-8	18	32	3.4	25-30	30	58	7.8
<i>wks.</i>							
3-5	5	11	3.2	30-35	18	39	8.0
5-10	18	27	4.7	35-40	14	29	8.9
10-15	34	60	5.5	40-45	12	23	8.2
15-20	36	68	6.5	45-50	7	10	9.3

* A period represents a single 24 hour determination for infants under 8 days of age, the average of two or three 24 hour determinations for infants under 5 weeks, and a 3 day average for all other infants.

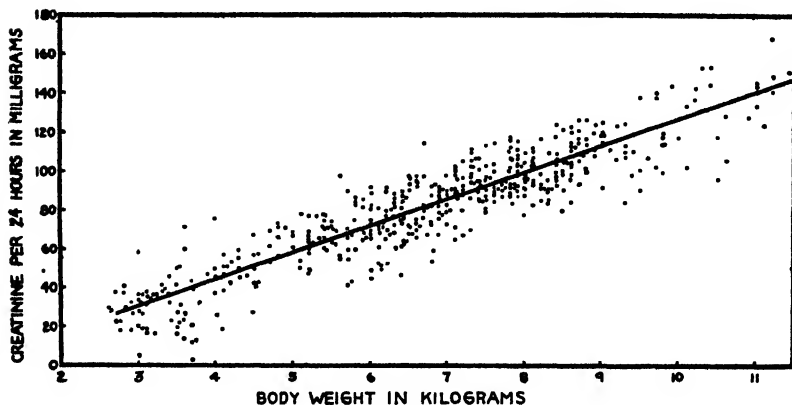


FIG. 1. The relation of daily creatinine excretion to the weight of the infant. The formula for the regression line is given in Table II, A.

the samples with picric acid for 30 minutes at 15 pounds pressure (3). Nitrogen was determined in food and excreta by the Kjeldahl-Gunning method (4). The values obtained are shown graphically in Figs. 1 to 4.

Creatinine

In Fig. 1, the creatinine data from the individual periods of study are plotted against the weight of the infants. Fig. 1 shows the consistent increase in creatinine excretion as the child increases in size and also the range of variability of creatinine excretion by infants of a given weight. In general, the values observed agree well with those reported by others studying infants fed similar diets (5-7).

TABLE II
Results of Statistical Analyses of Creatine and Creatinine Excretion of Infants

<i>Y</i>	<i>X</i>	Coefficient of correlation, <i>r</i>	Probable error*	Coefficient of alienation	Regression line of <i>Y</i> on <i>X</i>
<i>A</i>					
Creatinine-weight		0.9056 ± 0.0055	0.424		$Y = 13.555X - 9.334$
Creatinine-length		0.893 ± 0.0065	0.450		" = $3.233X - 123.947$
Creatinine-age		0.8333 ± 0.0094	0.553		" = $1.716X - 46.774$
<i>B</i>					
Creatine-weight		0.7645 ± 0.0128	0.645		$Y = 12.648X - 17.788$
Creatine-age		0.7313 ± 0.0144	0.682		" = $1.709X - 31.084$
<i>C</i>					
Creatinine + creatine-weight		0.8849 ± 0.0067	0.465		$Y = 26.776X - 30.2321$

* The "probable error" is such a number that the chances are even that the true coefficient of correlation in each case lies in the interval $r \pm \text{P.E.}$; or the chances are about 22 to 1 that the true coefficient of correlation lies in the interval $r \pm 3 \text{ P.E.}$

The data shown in Fig. 1 have been analyzed statistically and the correlation coefficients of creatinine with weight, length, and age of the infants determined. The results are shown in Table II, A. The correlation coefficients of creatinine with both weight and length are excellent and nearly identical, that with age slightly less good. As the daily creatinine excretion increased steadily throughout the period of infancy, it is to be expected that the three coefficients would be somewhat similar.

The creatinine-weight coefficient, 0.9056 ± 0.0055 , is unusually high for biological data and when one considers the impossibility of obtaining urine collections in exact 24 hour specimens from infants, the coefficient of alienation is surprisingly low. It may therefore be concluded that the factor chiefly responsible for the quantity of creatinine excretion is a function of body weight.

Length was considered by Daniels and Hejinian (6) as preferable to weight as a reference for creatinine comparisons among infants. The correlation between creatinine and length of the infants studied here was almost equal to that between creatinine and weight; the coefficient of alienation was slightly larger. The similarity of coefficients can be explained on an anatomical basis. Length is a function of skeletal growth. The skeleton remains at approximately the same percentage of body weight throughout life; the musculature is a constant proportion of body weight throughout infancy (1). During the period studied, then, both the musculature and the skeleton would remain in the same proportion to each other. Creatinine as a measure of quantity of muscle would thus show almost as good correlation with skeletal weight (measured by length) as with the weight of the body as a whole. The actual coefficients obtained show that this relationship holds, but in view of the probable causes, the creatinine-weight comparison seems preferable as being the more direct relationship.

The lower correlation coefficient of creatinine and age, compared with the others, is considered indicative that the apparent correlation should be ascribed wholly to the increasing weight rather than to any cause affected more directly by age. As a further test, the correlation coefficient of creatinine per kilo and age was determined. For this analysis, the data from the new born infants were excluded. The coefficient obtained, 0.083 ± 0.034 , within 2.5 times the probable error of zero, shows conclusively that during the 1st year creatinine excretion is not affected by the age of the infant.

The very close correlation between creatinine and weight indicates that the effect of exogenous factors is either constant or negligible. The exogenous factors of chief interest are the intakes of creatine and creatinine from milk. Human milk and cow's milk contain approximately equal amounts of creatine and creati-

nine (8). The average daily milk intake of the new born babies was 50 cc. for the first 2 days and 340 cc. for the 6th to 8th days, equivalent to an intake of total creatine-creatinine increasing from 0.6 mg. per kilo to 4 mg. per kilo. The average creatinine excretion increased from 8.6 to 10.5 mg. during this period. The collection error of the first 2 days of life, however, was relatively large, as the average 24 hour urine was less than 25 cc. The average milk intake per kilo of all the infants increased rapidly until a maximum was attained when the infants were about 4.5 kilos in weight. It then decreased throughout the remainder of infancy until at 1 year of age the average per kilo intake was 25 per cent less than the maximal. The maximal creatine-creatinine intake was 6 to 7 mg. per kilo. The amount of creatinine excreted increased from 8.6 mg. per kilo at birth to 12.5 mg. per kilo when the infant weighed about 5 kilos, after which the excretion per kilo remained constant. The quantity of intake of creatine and creatinine, therefore, exerts no consistent effect upon the quantity of creatinine excreted by the infants.

Analytical values of creatine content of muscle in infancy are variable. Rose (9) found the creatine content of the musculature in a new born infant to be 190 mg. per 100 gm. Denis (10) found variable amounts, averaging over 300 mg. per 100 gm. for infants about a year old. It seems probable that the creatine content of tissues increases during later infancy, whereas during this period the creatinine excretion per kilo remains constant.

No relation was observed between muscular activity as measured by achievement and the per kilo creatinine excretion.

It seems clear from the preceding discussion that the chief factor responsible for creatinine excretion is a function related to body weight and presumably more directly to the muscle weight. Scammon (1) found that the musculature is approximately 25 per cent of the body weight at birth and grows at a rate slightly lower than that of the body as a whole throughout the 1st year of life. These data were obtained early in the century and the infants studied were presumably fed human milk or cow's milk, diluted. The new born infants of the present study were fed human milk; those above 2 weeks of age were given cow's milk, undiluted. The nitrogen retentions of the latter group were much higher than those reported for infants fed human milk. Does this increased

retention of nitrogen change the percentage of body weight due to musculature and does it also alter the characteristics of infantile growth; that is, does the percentage of body weight due to musculature increase throughout the period of infancy?

To answer these questions comparison was made of the creatinine excreted per kilo by infants given the two types of feeding. The average creatinine excretion for the 1st week of life was 10 mg. per kilo, and one older infant fed human milk excreted an average of 9.9 mg. per kilo. Values observed by others for healthy infants fed human milk varied from 8 to 12 mg. per kilo (5, 11),

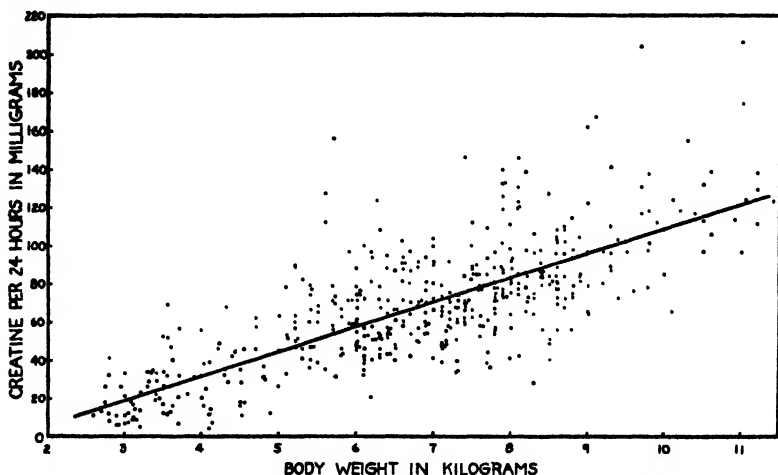


FIG. 2. The relation of daily creatine excretion to the weight of the infant. The formula for the regression line is given in Table II, B.

with an average of 9.8 mg. per kilo. The value of 10 mg. per kilo therefore may be assumed as an approximate average creatinine per kilo excretion for infants given human milk.

The infants of this study who were fed cow's milk were given this food with its higher protein content at 10 to 14 days of age. Before the 5th week of life, the average excretion of creatinine had increased to 12 mg. per kilo and after the 5th week remained approximately constant at 12.5 mg. per kilo even though the per kilo nitrogen intake decreased slowly thereafter (Fig. 3). One may thus conclude that a marked increase in protein intake in infancy results in a rapid increase in percentage of muscle tissue

up to a definite point, after which the proportion of muscle to body weight tends to remain constant as long as the dietary regimen is maintained. If one assumes that a creatinine excretion of 10 mg. per kilo represents a musculature 25 per cent of the total body weight, then an average creatinine of 12.5 mg. per kilo would indicate that the musculature of these infants represents about 30 per cent of the total weight. From the appearance of the infants, this increase in percentage of muscle seems to be at the expense of water and fat.

The average creatinine per kilo of the individual infants above 5 weeks of age varied from 10 mg. per kilo for the obese to 14.5 mg. per kilo for the very slender infants.

In conclusion, the data tend to show that the quantity of creatinine excreted by normal infants depends almost solely upon the quantity of musculature. The infant tends to maintain the musculature at a constant proportion of body weight throughout the 1st year of life, but the exact proportion maintained is somewhat higher in infants fed a high protein diet than in those fed human milk.

Creatine

The creatine-weight values are plotted in Fig. 2 in the same manner as the creatinine values of Fig. 1.

Each infant studied always excreted creatine. It is obvious from Fig. 2 that the creatine excretion of infants, like that of creatinine, increases steadily as the infant grows. It is also obvious that the quantities excreted daily are far more variable than those of creatinine. A part of this variability can be ascribed to method, but only a part. Whereas it was always observed that two infants of the same weight and body build excreted approximately the same amounts of creatinine daily, the same was not true for creatine excretion. Most of the infants habitually excreted somewhat less creatine than creatinine, six babies excreted only about half as much creatine as creatinine, and twelve consistently excreted as much or more creatine than creatinine. Four infants excreted more creatine than creatinine in early infancy, but after about the 20th week the creatinine excretion consistently exceeded that of creatine. The above factors, as well as the fact that the period to period variation in creatine excretion

was greater than that observed with creatinine, are responsible for the wide range of creatine values observed.

The data have been analyzed statistically with the results shown in Table II, B. The coefficients of correlation of creatine with

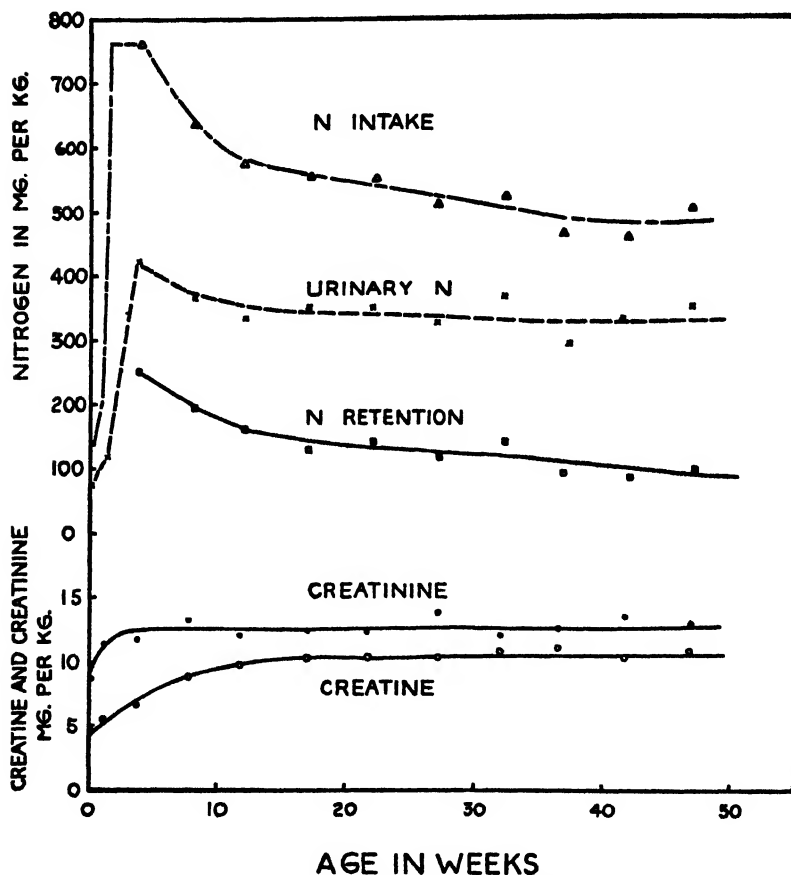


FIG. 3. The relation between nitrogen metabolism and creatine excretion. The arithmetic means for nitrogen per kilo and creatine and creatinine per kilo are calculated for 5 week intervals of age.

weight and age are fair, but the coefficients of alienation are almost equal to those of correlation. It seems that neither weight nor age alone is a predominating factor in determining the degrees of creatinuria in infancy.

Certain exogenous factors are known to affect creatine excretion. Of these, the most important for this study are the intakes of protein and creatine. To study these, the arithmetic means of the per kilo nitrogen intake, urinary excretion, and retention, and the excretion of creatine per kilo have been calculated for the new born period and for each succeeding 5 week period of infancy. The resulting curves are shown in Fig. 3. The creatine excretion, which was lowest at birth (4.6 mg. per kilo), remained at this value until the end of the 1st week, after feeding was well established. It then rose steadily to more than double the original value, or 10.5 mg. per kilo, a level which was attained between 15 and 20 weeks of age, and thereafter maintained throughout the remainder of infancy. The nitrogen intake of the infants was increased more than 3-fold upon institution of the feedings of cow's milk at the end of the 2nd week of life. The intake per kilo decreased slowly after the 5th week. The intake of exogenous creatine also reached a maximum before the 5th week and paralleled the nitrogen intake thereafter. Alterations in the creatine intake, like those of creatinine, were not paralleled by changes in creatine excretion. Similarly, no definite relationship could be observed between creatine excretion and any phase of nitrogen metabolism, intake, retention, or catabolized nitrogen, expressed as urinary excretion.

These results are in apparent contradiction to the findings of others (2). Such studies, it may be noted, have been of short duration. The infants of this study had been given the high protein feedings for 10 days to 2 weeks or more before the creatine excretion was studied. Unpublished studies on older children in this laboratory have indicated that a large part of the added creatinuria induced by a sharp increase in protein intake is a temporary phenomenon. It seems highly probable therefore that the excess creatinuria caused by increased protein intake of infants is also largely transitory. Whether all of it is transitory can only be determined through the study of a group of infants fed human milk for a period of several months. It has been possible for us to study only one such infant. His average excretion of creatine was 4.7 mg. per kilo, an amount similar to that of the new born infants and below that of any other older infant studied. He was given cow's milk for a period of 1 week, where-

upon the creatine excretion increased to 7.8 mg. per kilo, an initial increase lower than the average, but within the range, of the creatine excretion of the large group. Unfortunately it was not possible to study this infant further. Other studies bearing on the question include the report of Marples and Levine (7) quoting the creatine excretions of eight infants fed high protein diets. Seven of the babies excreted amounts of creatine comparable to those reported in this study, but the eighth, a month-old infant, excreted habitually less than 2 mg. per kilo daily. Ellinghaus, Müller, and Steudel (12) studied the total creatine-creatinine excretions of three infants given first a cow's milk formula, then human milk. The nitrogen intake was halved during the period when human milk was fed, but the total creatine-creatinine excretion was slightly increased. It seems possible that the creatine excretion of infants given a high protein diet may average somewhat greater than that of infants fed human milk, but certainly protein intake is not a major factor involved in determining the creatine excretion.

A comparison of the creatine and creatinine curves of Fig. 3 is of interest. Age is definitely not a factor in determining creatinine output per kilo, as was shown by the correlation coefficient, zero. The difference in values obtained for the new born and the older infants is explained as a true difference in quantity of musculature, due to differences in protein intake. To find the correct regression curve of creatine per kilo on age, however, one must assume an exponential law. Thus, while it may be concluded that age is not a factor in determining creatinine excretion, the same statement cannot be made for creatine.

The differences in quantity of creatine excreted per kilo by the individual infants of the same age and weight and under the same dietary regimen are further indication that the chief factor controlling creatinuria is endogenous. Of such factors which have been considered as influencing the creatine excretion, those most pertinent to the period under discussion are the creatine content of muscle (9, 10), the relative maturity of muscle (2), the carbohydrate metabolism of muscle (13), and the amount of thyroid hormone (13, 14).

The creatine content of muscle has been discussed under creatinine. Its relationship to creatine excretion seems no more obvious than that with creatinine excretion.

The creatinuria of infancy and childhood has been explained as the result of a low saturation point of the immature musculature for creatine (2). The continuous increase in creatine excreted per kilo during the early part of infancy is evidence against this explanation, as are also the findings (7, 15) that prematurely born infants often excrete no creatine, and that their creatine excretion remains lower for several months after birth than that of infants born at term.

Animal experiments (16) tend to show a definite connection between thyroid activity and carbohydrate metabolism, particularly glycogen content, of muscle. As far as the period of infancy is concerned, little is known definitely about either the glycogen content of muscle or the development of thyroid function (17). Such evidence as we have concerning the latter may be interpreted to indicate that thyroid secretion exerts a controlling influence over creatine metabolism during this period of life. The low creatine excretion of prematurely born infants may be correlated with the finding of Talbot (18) that these infants have a basal heat production definitely lower than that of infants born at term. Even at 4 months of age both the amount of creatinuria and the basal metabolic rate are lower for the prematurely born than for the full term infant. It is known that cretins excrete less creatine than normal children (14) and that creatine excretion is increased on institution of thyroid therapy. Creatinuria is increased also in cases of hyperthyroidism.

It was not possible to determine the basal heat productions of the infants reported in this study. From the data reported by Talbot (18) and the average weights of the babies of the different age groups studied (Table I), the average heat production in calories per kilo has been estimated for each age group. The resulting curve, together with that of creatine excretion per kilo, is shown in Fig. 4. The heat production in calories per kilo and the creatine excretion per kilo both increased during early infancy, and both remained approximately constant during later infancy. Although the relation between the two is not linear, both follow the same type of exponential law. Differences in relative activity of the thyroid might well account for the noticeable differences observed in relative quantities of creatine excreted by the different infants. It does not explain as readily the shift from high to lower creatine excretion observed in four infants at about 20 weeks

of age, unless one assumes an unusual residual supply of maternal hormone in these infants.

The findings in general tend to exclude exogenous factors from responsibility for the quantity of creatine excreted in infancy. A high protein intake may possibly increase the excretion of creatine, but no direct parallelism is observed between the level of protein intake and creatine excretion. The evidence presented indicates that the factor responsible for creatinuria is endogenous and supports the theory that activity of the thyroid may be directly or indirectly one of the chief factors concerned during infancy.

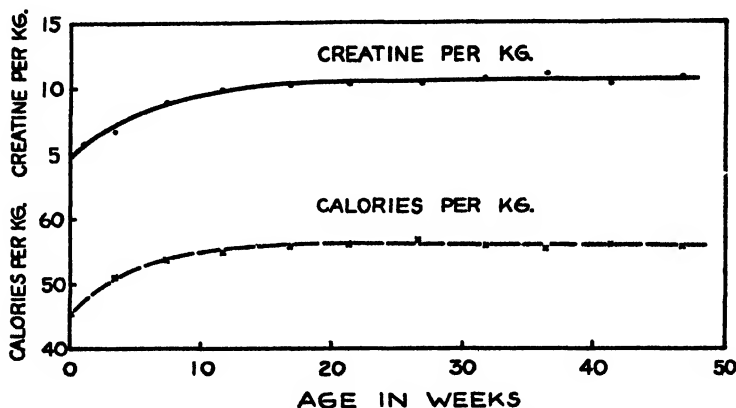


FIG. 4. The creatine excretion per kilo and the basal heat production per kilo calculated for infants of the same average weights (data from Talbot (18)).

Creatine and Creatinine

Harding and Gaebler (19) from a study of older children concluded that the excretion of creatine plus creatinine per kilo of body weight is a constant when the protein intake is high, and is the same for children as for adults. The sum of the average creatine and creatinine values per kilo for the infants of the present study increased from approximately 15 mg. at birth to 22.5 mg. after about 18 weeks of age. The latter value is similar to those observed in children by Harding and Gaebler. Average values for individual infants above 18 weeks of age, however, varied from 18 to 28 mg. per kilo, a difference of more than 50 per cent. The correlation coefficient of total creatine (as creatinine) and weight

(Table II, C) falls between that of creatinine-weight and creatine-weight, as would be expected of a fortuitous relationship. The creatine and creatinine curves of Fig. 3 also indicate no relationship between the two substances.

The creatine and creatinine excretions may be considered as manifestations of two different phases of muscle metabolism. The amount of creatinine excreted depends solely upon the quantity of muscle tissue, whether the individual is a new born infant or an adult. The creatine excretion, on the other hand, seems to be associated with some other phase of muscle metabolism, not directly related to quantity of muscle tissue, but more closely linked with hormonal control. The two phases of muscle metabolism are neither parallel nor reciprocal during early infancy; therefore probably at no time are they closely interrelated.

SUMMARY

This report summarizes the data from approximately 500 studies of creatine and creatinine excretion of infants from birth to 1 year of age. The quantities of both creatine and creatinine excreted increase throughout the entire period studied. The creatine excretion is far more variable than that of creatinine.

It is concluded that the creatinine excretion of infants is dependent practically in entirety upon the quantity of musculature. Infants fed high protein diets have a somewhat higher percentage of the body weight as muscle than infants fed human milk.

No consistent relationship is observed between the quantity of creatine excretion and creatine intake, or between creatine excretion and any phase of nitrogen metabolism.

Relative immaturity of muscle does not seem to be a factor in determining quantity of creatinuria.

The data have been interpreted as indicating that thyroid activity may be directly or indirectly the principal factor concerned in determining the quantity of creatine excretion of infants.

During early infancy, creatine and creatinine excretions neither parallel each other nor show any reciprocal relationship; this is considered evidence that the two substances represent different phases of muscle metabolism, not closely interrelated.

The authors are indebted to Dr. E. D. Plass of the Department of Obstetrics for permission to study the new born infants, and to Mr. Abraham Oleshen of the Department of Mathematics for the statistical analysis of the data.

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STUDIES ON THE CONSTITUTION OF INSULIN*

II. FURTHER EXPERIMENTS ON REDUCED INSULIN PREPARATIONS

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The study of the course of reduction of insulin by thioglycolic acid at about pH 2 has made possible the preparation in dry state of insulin samples of a varying degree of reduction (1). Chemical and physical measurements conducted on these preparations demonstrated that incipient denaturation of the protein occurs when the reduction has proceeded for 150 minutes under the experimental conditions selected. The product corresponding to this reduction period has been designated as reduced native insulin. The sulfhydryl content of these preparations indicates that a reduction of one or two disulfide groups of the hormone has occurred. Furthermore, the pharmacodynamic activity has decreased approximately 50 per cent. These results suggested the working hypothesis that one or two dithio ($-S-S-$) linkages in insulin represent activating groups; i.e., regulate the functioning of the active center, the nature of which is still obscure. The experiments described in the present communication were designed to test the validity of this hypothesis. The reduced native insulin preparations have been studied in detail with respect to their chemical and physical properties. If the hypothesis is tenable, it would be expected that such experiments will not reveal any significant alterations in the properties of the hormone molecule other than the previously reported formation of a small number of free $-SH$ groups. It is realized that the 50 per cent loss in physiological activity of the reduced insulin preparations

* The expenses of this investigation were defrayed in part by a grant from the Committee on Scientific Research, American Medical Association.

might also be accounted for by the possibility that one-half of the original insulin has remained unchanged.

EXPERIMENTAL

Insulin Preparations

The insulin used in the present studies was highly purified, amorphous insulin prepared by Eli Lilly and Company; the product had an activity of 20 to 22 units per mg. The reduction with thioglycolic acid and the subsequent removal of this reagent were carried out as described in the previous publication (1). A reduction time of 150 minutes at 30° and at pH 1.85 ± 0.05 was employed throughout.

Chemical Analyses

The reduced insulin preparations were analyzed for total sulfur, amino nitrogen, and tyrosine content. The total sulfur determinations were carried out by preliminary oxidation of the protein with nitric acid, followed by treatment of the solution with Denis' reagent (2). The amino nitrogen determinations were performed in a Van Slyke gasometric apparatus (3). Tyrosine analysis was conducted essentially as described by Herriott (4). The insolubility of insulin in the reaction mixture made it necessary to add concentrated urea solution (100 gm. of urea dissolved in 100 cc. of water) to make a final urea concentration of approximately 6 M. The color was permitted to develop in the reaction mixture for 5.5 hours at room temperature. The color intensity was then measured in the photoelectric colorimeter used in the previous work (1). The instrument was calibrated with solutions of pure *l*-tyrosine. It should be noted that in these determinations relative rather than absolute tyrosine values are obtained (4). Inasmuch as only the *relative* tyrosine content of oxidized and reduced insulin is of interest in the present study, the magnitude of the correction factor, obtainable from analysis of insulin hydrolysates for tyrosine, need not be considered. The results of the chemical analyses are given in Table I.

It is evident from Table I that the values for the original and reduced preparations agree satisfactorily. This indicates that treatment of insulin with thioglycolic acid, under the defined

experimental conditions, has not appreciably affected the total sulfur, the amino nitrogen, or the tyrosine content of the hormone.

Physicochemical Studies

It has previously been shown (1) that insulin preparations treated with thioglycolic acid for 150 minutes have a viscosity and solubility not markedly different from the original amorphous insulin. From this it has been concluded that the protein after this treatment is still essentially in the native state.

TABLE I
Chemical Analyses of Oxidized and Reduced Insulin

	Total sulfur	Amino nitrogen	Tyrosine
	<i>per cent</i>	<i>per cent</i>	<i>per cent*</i>
Insulin, Lilly† (—S—S— insulin)	3.20	0.93	10.92
	3.30		10.30
	3.26	0.92	9.55
Reduced insulin‡	3.11	0.87	10.35
	3.20	0.86	9.75
			9.80
			9.00

* The correction factor of Herriott (4) was not applied. The higher chromogenic value obtained in the present study is due to the presence of urea in the reaction mixture.

† The analytical values for oxidized insulin are corrected for moisture. The reduced insulin preparations, as isolated, are water-free.

‡ Each value for reduced insulin was obtained on fresh products prepared by identical procedures.

Molecular Weight—The study of insulin in the ultracentrifuge by Sjögren and Svedberg (5) has led to the conclusion that the molecular weight in the pH range of approximately 4.5 to 7.0 is 35,100. At lower and higher pH values, the molecule is reversibly dissociated into smaller units. It was of interest to ascertain whether the reduction process employed in this study results in a scission of the hormone molecule; in other words, whether the dithio linkages which are opened by the reduction serve as bridges between smaller aggregates. Professor The Svedberg kindly consented to conduct a comparative study of the behavior of the original amorphous insulin and the reduced

native preparation in the field of the ultracentrifuge. The results of preliminary measurements were as follows: The reduced product has practically the same sedimentation constant as the original oxidized preparation. The homogeneity is also similar in the two products. However, neither of the preparations is completely homogeneous. At the alkaline and acid limits of the stability range, the reduced product begins to dissociate earlier than the oxidized compound. The exact figures will be published by Professor Svedberg.

Isoelectric Point—The isoelectric points of reduced insulin and of the original amorphous insulin were determined by means of a Northrop-Kunitz (6) type of cataphoresis cell.¹ In each experiment the protein was adsorbed on collodion particles. The isoelectric point of both the reduced and the original insulin preparations was found to be 5.35 ± 0.02 pH units. This agrees well with the results obtained for amorphous insulin by previous investigators (7, 8).

Absorption Spectrum—Insulin shows specific light absorption in the ultraviolet range of the spectrum. Kuhn, Eyer, and Freudenberg (9) conclude that the absorption band with a peak at 2750 Å. is due to the tyrosine and, to a small extent, also to the cystine present in the hormone. Their experiments show that, while insulin can be inactivated without affecting the absorption spectrum, alteration of the absorption spectrum is always associated with inactivation.

Solutions of the original and of the reduced insulin containing 50 mg. of protein in 5 cc. of redistilled water (pH of solution approximately 2.5) were prepared. The region from 2200 to 4000 Å. was measured with a Hilger No. E-316 quartz spectrograph and a Spekker photometer; an iron spark served as light source. The plates were evaluated by the match point method.² The resulting curves showed a very similar absorption pattern for the two preparations. The position of the absorption band near 2800 Å. is about the same within the limits of error of the method.

¹ The authors wish to thank Dr. Leslie F. Nims for conducting these determinations.

² The measurements were carried out by Dr. Robert T. Armstrong, Department of Chemistry, Massachusetts Institute of Technology.

Reoxidation of Reduced Insulin—Inasmuch as the results obtained indicate that the only detectable change produced by the reduction consists in the appearance of a small number of sulfhydryl groups, an attempt was made to reverse this change by oxidation. It is known that thiols of the type of cysteine or glutathione will react with atmospheric oxygen only in the presence of small amounts of heavy metals which act as catalysts (10). It has been demonstrated that cysteine under these conditions may be quantitatively converted to cystine. In this reaction, copper, iron, and manganese are effective.

Reduced insulin was dissolved in redistilled water and 0.1 N NaOH was added until pH 7.55 was attained. 3 cc. portions of this solution containing 97.5 mg. of reduced insulin were placed in Warburg vessels in an air atmosphere. Up to 30 minutes, there was no oxygen uptake at 30°. The addition of 2×10^{-4} and 6×10^{-4} mg. of Cu (as CuSO_4 solution) caused a small oxygen uptake within 65 minutes (11.2 and 7.75 c.mm. of O_2 respectively). The vessels were then opened and the copper concentration was increased to 2×10^{-2} mg. of Cu and 6×10^{-2} mg. of Cu respectively. This resulted in an increased initial rate of oxidation; the solution containing the lower copper concentration absorbed 38 c.mm. of oxygen, while that containing the higher copper concentration absorbed 45 c.mm. of oxygen within 240 minutes. A number of similar experiments at hydrogen ion concentrations varying from pH 8.25 to 8.7 with varying amounts of copper, iron, and manganese (added as the sulfates) were conducted. A typical experiment performed at pH 8.25 with copper and iron as catalysts is shown in Fig. 1. With a sufficient metal concentration (considerably higher than that required in similar experiments with cysteine) the oxygen uptake was usually almost completed after 3 to 4 hours. The analysis of the experimental mixtures demonstrated that the —SH groups had disappeared almost completely. The oxygen uptake closely approximated that calculated to be required for the conversion of the thiol groups present in the reduced insulin preparations to the disulfide form. For example, in one experiment at pH 8.7 with a copper concentration of 6×10^{-2} mg., 91 mg. of reduced insulin absorbed 58.2 c.mm. of oxygen in 240 minutes. The preparation used contained 1.45 per cent cysteine equivalents. The calculated volume of oxygen

required for the conversion to cystine is 60.7 c.mm. Colorimetric analysis at the end of the experiment revealed only residual traces of cysteine. Iron and copper were found to be more efficient as catalysts than manganese when these metals were tested under similar conditions. At pH 2.6, reduced insulin, like cysteine, is not oxidized under similar experimental conditions.

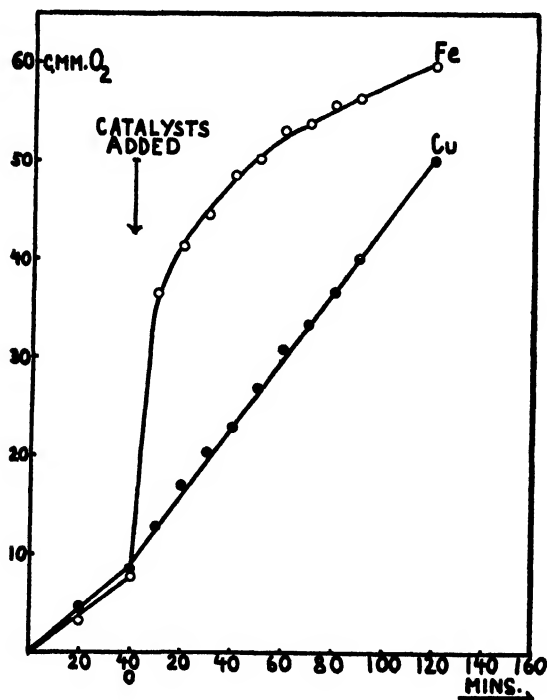


FIG. 1. Course of oxidation of reduced insulin by air with and without iron and copper as catalysts. The abscissa shows time in minutes; the ordinate, uptake of oxygen in c.mm., as observed in the Warburg manometric apparatus. 6×10^{-2} mg. of Fe and 6×10^{-2} mg. of Cu were used in this experiment.

It has therefore been possible to demonstrate the chemical reversibility of insulin reduction. It remained to be determined whether this reoxidation was accompanied by a restitution of the entire physiological activity. For this purpose, the two re-oxidized products obtained in the experiments shown in Fig. 1 were recovered from the experimental mixtures. They were then

sent in sealed ampules to the Lilly Research Laboratories where they were assayed under the supervision of Mr. George B. Walden. The mouse convulsion method of assay was used. The results of the bioassay demonstrate that both samples possess approximately 1 per cent or less of the physiological activity of the original oxidized, amorphous insulin; *i.e.*, 0.2 unit, or less, per mg. of solids. Contrary to expectation, therefore, the reoxidation not only failed to restore the full physiological activity, but yielded a product in which the residual activity present in reduced native insulin (10 to 12 units per mg.) has been almost entirely abolished.³ The experiment was repeated with the substitution of the original, amorphous insulin for the reduced preparation. The pH was 8.56. Within 180 minutes, the oxygen uptake in vessels containing insulin, with and without the addition of copper, was only 4 c.mm. The bioassay of the recovered products showed an activity of 16 units per mg. for the sample without the catalyst, and 13 units per mg. for the sample to which copper had been added.⁴

SUMMARY

The chemical analyses and the physicochemical measurements reported in the present paper have not revealed any significant differences between the original and the reduced native insulin preparations other than the previously described presence of a small number of free sulfhydryl groups in the latter. The two products are practically identical with respect to their content of tyrosine, free amino groups, and total sulfur; also with respect to their molecular size, isoelectric point, and viscosity. The ultra-violet absorption spectra show a similar pattern. Reoxidation of the reduced product, under the conditions described, results in the disappearance of the free sulfhydryl groups, accompanied by the almost complete abolition of physiological activity. Treat-

³ It should be noted that the partial reactivation of reduced insulin reported by Freudenberg and Wegmann (11) was effected by a different method of reoxidation.

⁴ A similar control experiment with amorphous insulin, with iron as the catalyst, yielded a product with an activity of 14 to 15 units per mg. A total of 2160 mice was used for the bioassay of the three products obtained in these control experiments.

ment of the original insulin under comparable conditions leads to a loss of from 20 to 38 per cent of the physiological activity.

The authors wish to thank Eli Lilly and Company for continued assistance in this research, and to express appreciation to Mr. George B. Walden for conducting the bioassays in the Lilly Research Laboratories.

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THE CONCENTRATION AND PROPERTIES OF VITAMIN H

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The vitamin concerned in the present report is a relatively heat-stable component of the vitamin B complex, which is essential for growth and for prevention of erythredemic dermatosis in rats. It represents the residuum necessary in addition to vitamins B₁ and riboflavin to complete the growth-promoting activity of the vitamin B complex. For purposes of convenience in presentation it will be referred to here as vitamin H. The probable relation of vitamin H to other factors of the vitamin B complex is discussed at the end.

The relative vitamin H values of the source materials and concentrates derived from these were estimated on the basis of the growth rates of young and suitably standardized rats. White rats, 28 to 29 days of age, reared in families on diets consisting chiefly of milk and wheat, and weighing from 40 to 55 gm., were given *ad libitum* a diet of the following composition.

	per cent
Purified casein	18
Osborne and Mendel (1) salt mixture*.....	4
Agar.....	2
Cod liver oil	4
Corn oil.....	4
Corn-starch	68
	<hr/> 100

* Modified by the addition of copper sulfate.

After 1 week each animal was given in addition to this diet 20 micrograms of crystalline vitamin B₁ per day and a highly concentrated riboflavin preparation equivalent to 20 micrograms of crystalline riboflavin per day or 20 micrograms of crystalline

riboflavin. The rats receiving these addenda ceased to grow after a period of 2 to 3 weeks. One animal from each litter was continued on the vitamin B complex-deficient diet supplemented with the vitamin B₁ and riboflavin addenda and others were given, in addition, suitable daily quantities of the preparations to be tested for vitamin H activities. The control rats lost weight slowly and developed marked signs of vitamin H deficiency in 2 to 3 weeks after they had ceased growing. The vitamin H values are expressed in terms of the usual "growth units," a unit of the vitamin being carried by that quantity of a preparation which will support growth at the rate of 3.0 gm. per week over a 4 week test period. It was found that approximately 3 times the "unit" quantity of a vitamin H preparation was required to support a growth rate of 7 to 8 gm. per week. Animals which grew at a rate of 3 gm. or more per week did not show any of the characteristic signs of vitamin H deficiency.

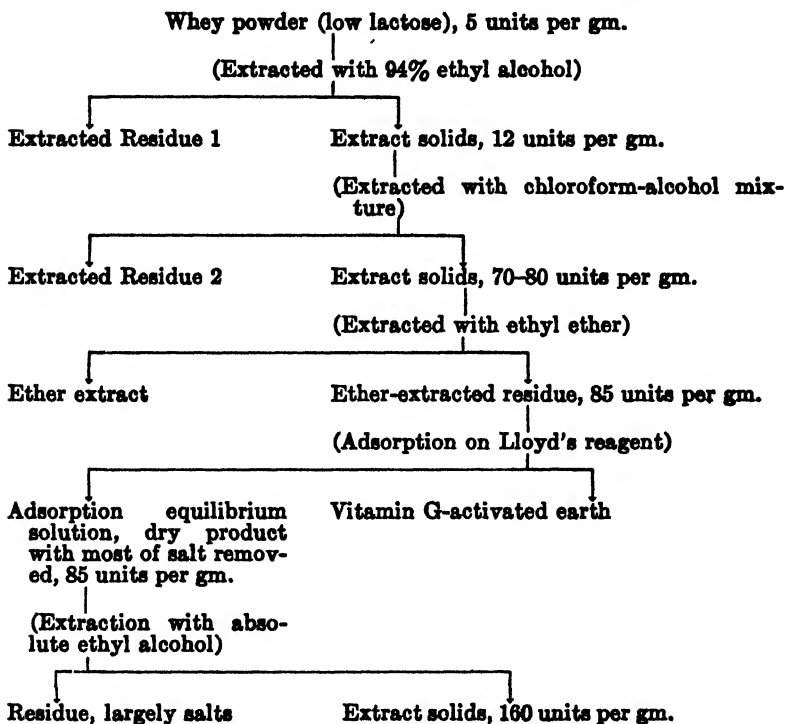
*Concentration of Vitamin H from Whey Powder¹
Low in Lactose*

A method for the concentration of riboflavin involving successive extractions of low lactose whey powder¹ with boiling ethyl alcohol (93 to 94 per cent by weight), with an alcohol-chloroform mixture, and with diethyl ether was described earlier (2). These initial steps in the process of concentrating riboflavin were found to concentrate vitamin H simultaneously. Following the extraction with diethyl ether, 5 gm. of the ether-extracted residue were suspended in 300 cc. of water to which were added 300 cc. of 2 M hydrochloric acid (acidities within the range of 0.2 M to 2.0 M served equally well) and 17 gm. of Lloyd's reagent. The earth, carrying practically all of the riboflavin, was separated from the adsorption equilibrium solution by centrifugation. The supernatant liquid was neutralized with sodium hydroxide and concentrated under reduced pressure (6 to 10 cm.) to about one-tenth of its original volume. The solution filtered from the deposit of sodium chloride was dried and tested for vitamin H activity. From 5 gm. of the ether-extracted residue carrying 85 units of vitamin H per gm., 3.8 gm. of total solids carrying 85

¹ This product was obtained through the courtesy of Dr. L. A. Rogers, Bureau of Dairy Industry, United States Department of Agriculture.

units per gm. were recovered from the adsorption equilibrium solution, corresponding to approximately 75 per cent recovery of vitamin H from the ether-extracted product. Extraction of this dry product with absolute alcohol yielded a concentrate (2.0 gm.) containing practically all of the vitamin H activity in

Diagram 1
Concentration of Vitamin H from Whey Powder



a concentration of 160 units per gm., somewhat greater than 30-fold the vitamin H potency of the low lactose whey powder.

A summary of the successive steps involved in concentrating vitamin H of low lactose whey powder together with the corresponding vitamin H values of the preparations is shown in Diagram 1.

Concentration of Vitamin H from Rice Polishings

Two separate samples of rice polishings were tested for their vitamin H values and were found to carry 12 and 13 units per gm. respectively. The concentration procedures are outlined in Diagram 2.

Rice polishings were extracted with dilute sulfuric acid (pH 5.0). One lot of extract corresponded to 1 gm. of polishings per 10 cc. of extract, and another to 2 gm. of polishings per 10 cc. of extract. The acidulated extracts were treated with fullers' earth for removal of the vitamin B₁ incident to the Williams *et al.* (3) method for the isolation of vitamin B₁. The resultant vitamin B₁-spent filtrate² was used as a source material for the concentration of vitamin H. The neutralized vitamin B₁-spent filtrate was distilled under reduced pressure and dried before a fan at room temperature. The air-dried product carried 22 units of vitamin H per gm.

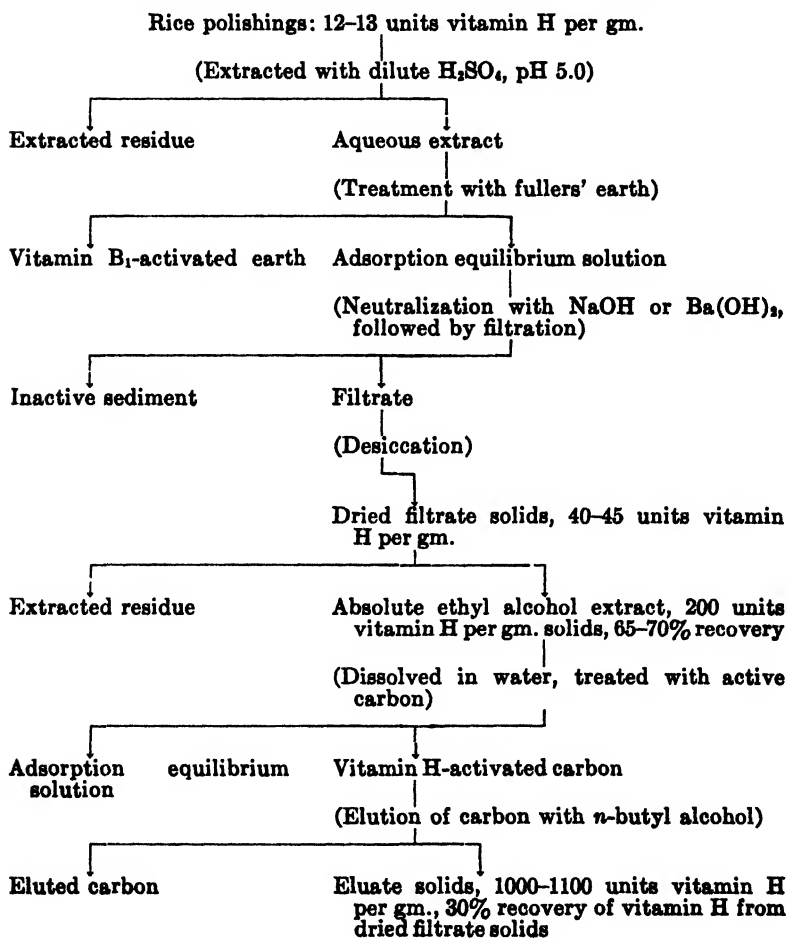
Neutralization of the vitamin B₁-spent filtrate with sodium or barium hydroxide was attended with deposition of 40 per cent of the solids of the extract. This sediment carried only an insignificant fraction of the vitamin H activity and henceforth was discarded. The air-dried solids of the resultant filtrate were used in subsequent steps of the concentration procedure. These solids from the two lots of vitamin B₁-spent filtrate carried 40 and 44 units of vitamin H per gm. respectively.

80 per cent ethyl alcohol, absolute ethyl alcohol, *n*-butyl alcohol, isoamyl alcohol, and acetone each purified by standard procedures were tested for their respective efficiencies in extracting and concentrating the vitamin H activity from the solids of the neutralized vitamin B₁-spent filtrate from which the inactive sediment had been removed. 25 gm. portions of solids were extracted three successive times at room temperature with 100 cc. portions of the respective extraction fluids. Each extraction was of 30 minutes duration, the extraction systems being continuously agitated by a mechanical shaker during this time. The combined extracts in each case were concentrated to a few cc. by distillation under reduced pressure (about 10 cm.), water being added inter-

² This product was furnished through the courtesy of Merck and Company, Inc.

mittently to remove the last traces of organic solvent. The concentrated extracts were dried over sulfuric acid or phosphoric anhydride. The respective vitamin H potencies and recoveries

Diagram 8
Concentration of Vitamin H from Rice Polishings



measured on the dry products were as follows: 80 per cent ethyl alcohol extract, 60 units of vitamin H per gm., 90 to 95 per cent recovery of the vitamin; absolute ethyl alcohol extract, 200 units

of vitamin H per gm., 65 to 70 per cent recovery of the vitamin activity; *n*-butyl alcohol extract, 240 units of vitamin H per gm., 30 to 40 per cent recovery of the vitamin activity; isoamyl alcohol extract, 300 to 350 units of vitamin H per gm., 30 per cent recovery of the vitamin activity and acetone extract, 420 units of vitamin H per gm. with 14 per cent recovery of the vitamin activity.

By treatment of the absolute alcohol extractives in a water medium with acid-purified, activated charcoal (norit), it was found that a large proportion of the vitamin H activity was adsorbed by the charcoal. An amount of absolute alcohol extractives corresponding to 25 gm. of the dried filtrate solids was dissolved in 50 cc. of water and treated with three successive portions (500 mg., 250 mg., and 250 mg.) of acid-leached, activated charcoal at temperatures of 6-8°. Separate samples of the vitamin H-activated charcoal were subjected to different elution processes. The elution media which were investigated included ethyl alcohol, ethyl alcohol and acetic acid mixtures, ethyl alcohol and pyridine mixtures, ethyl alcohol and benzene mixtures, *n*-butyl alcohol, and acetone. The elution media, in three successive portions, were refluxed with the vitamin H-activated charcoal for 10 to 15 minutes. The spent charcoal after each treatment was filtered off while still hot and the combined eluates distilled with an excess of water to a very small volume. The preparations were dried over sulfuric acid and then over phosphoric anhydride in a vacuum desiccator. Ethyl alcohol or a mixture of ethyl alcohol and glacial acetic acid was least effective as an elution medium. The following elution media were effective in removing the adsorbed vitamin H from the norit with resultant concentrations varying from 800 to 1100 units of vitamin H per gm. of dry solids and are here recorded in the ascending order of their effectiveness in concentrating vitamin H: acetone, ethyl alcohol and pyridine mixtures, *n*-butyl alcohol, and ethyl alcohol-benzene mixtures (mixtures of equal volumes). In the case of the ethyl alcohol-benzene mixture, the highest degree of concentration was effected only after the small quantity of water adhering to the charcoal was removed by a preliminary distillation with excess of benzene in the presence of the charcoal, the absolute ethyl alcohol being added subsequently and the elution mix-

ture refluxed in the usual manner adopted for the other elution media.

The manipulation of the eluates was very similar for each of the elution media. The details for one of these (*n*-butyl alcohol), as an example, were as follows: The solids obtained from an absolute ethyl alcohol extract of 50 gm. of the vitamin B₁-spent filtrate solids were taken up in 200 cc. of water and treated with three successive portions (1.0 gm., 0.5 gm., and 0.5 gm. respectively) of acid-leached activated norit. The combined vitamin H-activated norit portions were refluxed for 10 minutes each time with 180 cc., 100 cc., and 100 cc. respectively of freshly distilled *n*-butyl alcohol. The eluates were combined and distilled under reduced pressure (6 cm.) with an excess of water to a volume of 4 to 5 cc. The concentrated water solution of the eluate was dried, yielding 610 mg. of a preparation carrying from 1000 to 1100 units of vitamin H per gm. of dry solids. This preparation contained many clusters of tiny elongated crystals embedded in a brittle, straw-colored matrix. 1 mg. of this preparation administered daily to suitably standardized vitamin H test rats induced a growth rate of 3.4 ± 0.7 gm. per week over a 4 week test period; 2 mg. of the same preparation per rat per day induced a growth rate of 5.1 ± 0.4 gm. per week. On the basis of the calculation described earlier, the growth of 3.4 gm. per week would correspond to about 1.1 units of vitamin H and that of 5.1 gm. to about 2.0 units of vitamin H. The yield of product would indicate an approximate recovery of 30 per cent of the vitamin H activity from the 50 gm. of vitamin B₁-spent filtrate solids or approximately 45 to 50 per cent of the vitamin H activity from the absolute ethyl alcohol extract (of the vitamin B₁-spent filtrate solids) which had been treated directly with the charcoal.

Charcoal treatment of solutions of less purified concentrates of vitamin H was not satisfactory. The practise of refluxing the elution media in contact with the vitamin H-activated charcoal was an important factor in securing highly active concentrates.

DISCUSSION

A consideration of the properties ascribed to various factors of the vitamin B complex, the precipitate factor from liver reported by Elvehjem and coworkers (4), the "filtrate factor" (5-8), and

vitamin B₆, would indicate that vitamin H possessed a combination of the properties of more than one of these factors. All of these factors have been reported to be essential for the growth of rats. The basal ration used in the experiments here reported, with addition of vitamin B₁, riboflavin, and vitamin H concentrates, promoted the growth of rats and prevented the appearance of any gross signs of physiological abnormality in the animals.

The occurrence of the different factors of the vitamin B complex in natural food materials is quite similar. Halliday and Evans (9), however, have recently shown that the adsorption behavior of the different factors is considerably dependent on the nature of the source materials used for preparation of the extracts. Birch and György (10) have reported that vitamin B₆ is not adsorbed by norit at pH 6.0 but had previously found this vitamin in the Kinnersley and Peters (11) concentrate of vitamin B₁. Elvehjem and coworkers (4) have reported the precipitate factor to be concentrated on norit but encountered difficulty in removing it from the norit. Vitamin H was adsorbed by norit from previously purified preparations in neutral aqueous solution and could be removed by any one of several different organic solvents.

In comparison with vitamin B₁ and flavins, vitamin H from whey concentrates or from aqueous extracts of rice polishings was sparingly adsorbed by fullers' earth from aqueous acid solutions. A large proportion of fullers' earth will effect significant adsorption of vitamin H.

The presence of vitamin H, as distinct from vitamin B₁ and riboflavin, in an 80 per cent alcoholic extract of wheat (12) is confirmed in the work of Copping (13) on flavin and vitamin B₆ in cereals.

SUMMARY

A 30-fold concentration of vitamin H, a component of the vitamin B complex essential for growth and for the prevention of a severe erythredemic dermatosis in rats, has been obtained from whey powder by a series of extraction procedures interposed by treatment with fullers' earth. The concentrate carries neither flavins nor vitamin B₁.

A 60- to 90-fold concentration of vitamin H has been obtained

from rice polishings by procedures involving chiefly extractions and adsorption on and elution from charcoal. This concentrate was uncontaminated by either vitamin B₁ or flavins.

These concentrates carried the residuum necessary in addition to vitamin B₁ and riboflavin to complete the growth-promoting activity of the vitamin B complex.

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AN APPARATUS FOR THE MEASUREMENT OF THE METABOLIC RATE OF SMALL ANIMALS

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(Received for publication, March 12, 1937)

The variation in the metabolic rate during a 24 to 36 hour period has been studied in experiments to be reported later.

To permit the study of the metabolic rate of the rat a special device has been constructed.

Apparatus

The rate of O_2 consumption is measured continuously; the rate of CO_2 production during 45 minute periods. A movement recorder gives a continuous record of the grosser bodily movements. The atmosphere has the composition of air at a relative humidity of 85 per cent.

Measurement of O_2 Consumption—The rate of O_2 consumption is measured electrolytically. A diagram of the apparatus is given in Fig. 1. The animal is placed in the boat (*A*) which floats on a solution of NaOH (*B*). The pipe (*C*) connects the respiration chamber (*D*) to the electrolysis cell (*E*). In the electrolysis cell is the electrode (*F*) which is the oxygen-producing end of an electrolytic circuit.

The pressure of the air confined in the respiration chamber, the pipe, and the space above the liquid in the electrolysis cell is maintained constant. This is accomplished by the following mechanism.

The resistance to the flow of an electric current at the junction of a metallic and a liquid conductor in a circuit is largely a function of the area of the metal exposed to contact with the liquid. This fact is the basis of the measurement of the rate of oxygen consumption. When the animal in the respiration chamber consumes oxygen and produces carbon dioxide, the partial pressure

of the oxygen is reduced. The partial pressure of the carbon dioxide remains the same, since the expired CO_2 is absorbed as fast as it is produced by the solution of NaOH . The net result is a fall in total pressure inside the chamber below the external pressure and the electrolytic solution must rise up the tube of the electrolysis cell. This action increases the area exposed to the liquid. The flow of current through the solution is thus increased

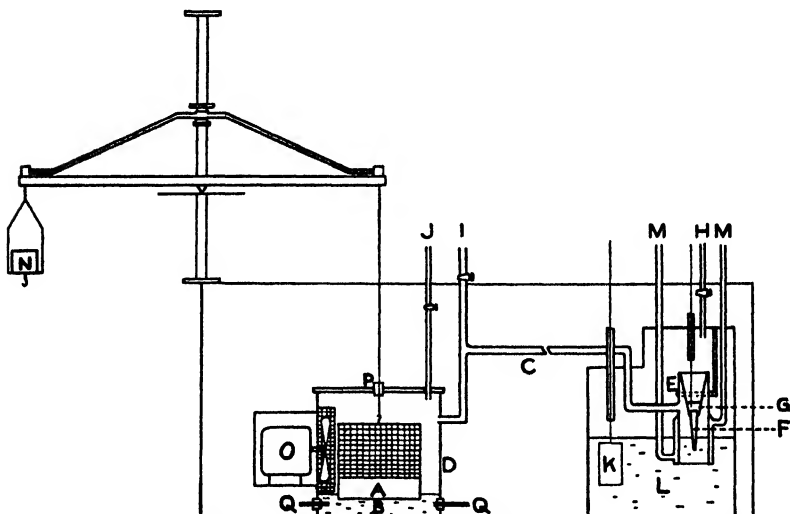
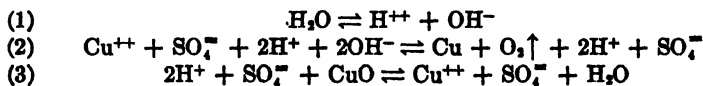


FIG. 1. Diagram of apparatus assembled. *A*, animal cage; *B*, NaOH solution for absorbing CO_2 ; *C*, pipe connecting electrolysis cell with respiration chamber; *D*, respiration chamber; *E*, electrolysis cell; *F*, electrode at which O_2 is produced; *G*, mercury contact; *H*, *I*, *J*, pressure-adjusting valves; *K*, negative electrode on which Cu is deposited; *L*, solution of CuSO_4 with a layer of CuO on the bottom; *M*, *M*, pipes of cooling circuit; *N*, counterweight for rat and animal cage; *O*, motor for fan which circulates air in the respiration chamber; *P*, oil seal allowing passage of wire support of the animal cage to the exterior; *Q*, tubes of NaOH circulation system.

and more oxygen is produced per minute. It follows that the liquid will rise to a point at which the rate of production of oxygen is equal to the rate at which the oxygen in the chamber is being consumed. The rate of current flow is measured by a recording ammeter. A continuous record of the rate of oxygen consumption is thus obtained. Any variations in this record follow variations in the oxygen consumption within a maximum lag of 15 seconds.

A labeled diagram of the electrolysis cell (*E*) is given in Fig. 1. Mercury fills the cup (*G*). It serves as a means of low resistance contact between the electrode and the rest of the circuit. The shape of the electrode (*F*) is an elongated blunt V. This shape gives a great change in area per unit distance of movement of the liquid surface without exaggerating small changes. Cold water to prevent overheating of the cell is circulated through pipes (*M*).

The electrolysis solution (*L*) consists primarily of a saturated solution of CuSO_4 . To this CuO is added after every experiment, so that there is always a thin layer of the insoluble material on the bottom of the jar (*F*). The reactions which take place in the presence of the current are:



The CuO is added to prevent the sulfuric acid concentration from rising too high and also to keep the solution saturated with CuSO_4 . Too high a concentration of H_2SO_4 would lead to a formation of persulfuric acid, thus detracting from the accuracy of the readings. It is apparent that copper is deposited on the negative pole (*K*) and that H_2 is not a product of the reaction. This is the reason for using CuSO_4 as the electrolyte, for, since no gases are produced at the negative pole, variations in barometric pressure can be excluded.

The recording ammeter is a product of the Esterline Angus Company. It is furnished with 0.05, 1, and 2 ampere shunts. Its scale is divided into 50 divisions; it is accurate to 1 per cent at any point on the scale except for values below 0.2 of the shunt value. One of the three shunts is used, depending on the amount of oxygen the animal is consuming. The smallest shunt possible is used.

The value of the readings in oxygen is easily determined by using the electrochemical equivalent and correcting to cc. per minute for 1 ampere. The method is $m = eit$, where m = the mass of substance deposited, e = the electrochemical equivalent, i = the current, t = the time in seconds. Values other than 1 ampere are simply multiples of 3.48 cc. per minute, when calculated on a basis of rate per minute.

Current is generated by a motor generator set. The voltage

on the field coils of the generator, and thus the voltage impressed on the electrolysis circuit, is regulated by a potentiometer circuit. This method furnishes a delicately adjustable voltage constant at the desired setting. Thus with a constant impressed voltage of suitable value the only change in current flow is due to changes in O_2 requirements of the animal.

The chamber is shown in Fig. 1. It is built of brass. The sides are of 1/36 inch brass sheets soldered to a welded angle brass frame. The bottom is $\frac{1}{8}$ inch brass plate. The cover, another $\frac{1}{8}$ inch brass plate, fits into a depression formed by the frame. The present and only satisfactory method of sealing is with hot paraffin painted on with a flat brush. Such a seal is renewed with each experiment, is water-proof, solid, and never wears out.

The electrolysis chamber is a 12 inch Pyrex jar with a cover of $\frac{1}{8}$ inch brass plate. A square housing containing the tubes leading up from the electrolysis cell is built onto the cover. A support let down from the plate holds the cell. The glass tube of the cell is soldered into the pipe with De Khotinsky cement.

The animal chamber (A) consists of a square boat made of copper with a cage wire mesh above. A galvanized zinc lid prevents condensation water from dropping into the cage. Supports and the method of hanging are shown in Fig. 1. A flat water bottle is placed on top of the animal cage.

Rapid circulation of the air is achieved by a *brushless* electric fan motor driving a fan. The motor (O) is enclosed in the motor chamber as shown in Fig. 1. The shaft protrudes through a hole just large enough to accommodate it. The fan guard prevents the animal cage from hitting the fan.

Measurement of Production of Carbon Dioxide—The rate of production of carbon dioxide is measured by the rate of change of the conductivity of the NaOH solution (B). 4 liters of solution are used. The number of gm. of NaOH dissolved can be varied to suit the age of the animal being studied. 20 gm. per 4 liters is the charge used for a 12 hour run on an adult rat. The charge is used until it becomes a 0.1 N solution of NaOH.

Sodium carbonate has one-third the conductivity of NaOH. By using a conductivity cell 30 cm. long made of $\frac{3}{8}$ inch bore glass tubing a very high initial resistance is obtained. It is of the order of 10,000 ohms. With the above charge 1 ohm change in resistance is equal to approximately 0.5 cc. of expired CO_2 .

Calculations and Method of Calibration—In calibrating this system a solution of NaOH of the concentration to be used in the experiment is placed in the respiration chamber. Carbon dioxide is added. The resistance is measured and the solution is analyzed for the amount of CO_2 it contains.

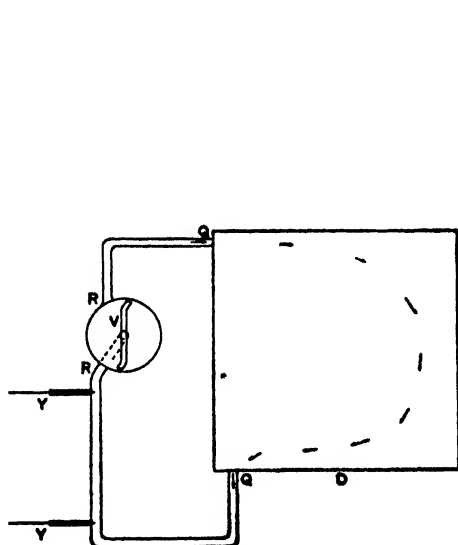


FIG. 2

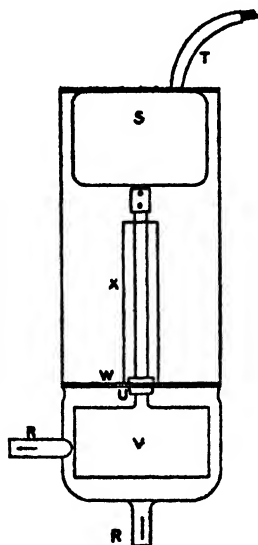


FIG. 3

FIG. 2. Schematic diagram showing the location of circulating pump-conductivity cell and NaOH reservoir in position. *D*, respiration chamber; *Q*, tubes of NaOH circulation system; *R, R*, inlet and outlet of centrifugal pump; *V*, vane of centrifugal pump; *Y, Y*, electrodes of conductivity cell.

FIG. 3. Diagram of centrifugal pump used to pass NaOH from reservoir through the conductivity cell. *R, R*, inlet and outlet of pump; *S*, motor; *T*, air-tight tube for motor leads; *U*, rubber bearing for vane shaft; *V*, vane of pump; *W*, glass plate; *X*, glass tube preventing splash from the vane from getting above plate *W*.

The graph of CO_2 concentration against conductivity, in the range used, is a straight line. Computations of CO_2 output involve only the multiplication of the change in conductivity found between two readings, by the slope constant for the line.

The slope of the calibration curve is unaffected by small changes in concentration of the NaOH solution. Thus values obtained need not be corrected for concentration changes induced by water condensation which is derived from the animals' respiration

Descriptions of Units in CO₂ System—The conductivity cell (*Y*, *Y*) is shown in Fig. 2. The centrifugal pump (*V*) for the circulation of the NaOH solution and the cell, are connected to the reservoir of NaOH in the respiration chamber as shown at *Q*.

The centrifugal pump is made of glass after a special design shown in Fig. 3. Its motor (*S*) is inclosed in an air-tight glass chamber. The motor shaft is connected through a rubber bearing (*U*) with the blade (*V*). NaOH is prevented from reaching the motor chamber by the glass plate (*W*) and the glass shaft tube (*X*). The wire leads to the motor pass through the air-tight seal (*T*). The NaOH circulates through the outlets (*R*).

The Wheatstone bridge was built from the simplified design given by Jones and Josephs.¹ A difference of 0.1 ohm in the resistance of the conductivity cell is easily measured by it.

All metal units in contact with the NaOH are protected by a thick layer of paraffin.

Recording of Movement—A record of the movements of the animal in an experiment on the metabolism of animals is important. Further, it should be possible to compare directly all movement and correlated change, if there is any, in the rate of oxygen consumption. In this device the movement recorder is sensitive to the grosser bodily movements. A record of these movements is written on the ammeter chart. A diagram of the movement recorder is given in Fig. 1. The weight (*N*) counterbalances the animal cage (*A*) and rat. The cage is suspended on a wire passing through the air-tight oil seal (*P*).

Operation of Apparatus—As soon as the animal has been placed in the respiration chamber, the lid of the chamber is sealed with paraffin. The production of O₂ is begun. The thermostat is filled and brought to the correct temperature. It is maintained at this point by thermostatic control.

In Fig. 1 are shown three pressure-adjusting valves (*H*, *I*, *J*). The two connected with the respiration chamber are used to allow the circulation of air through the chamber when the NaOH is being changed during a run of more than 12 hours duration. The valve on the electrolysis chamber is closed when the electrolysis solution has reached operating temperature.

The apparatus as designed is essentially automatic, requiring

¹ Jones, G., and Josephs, R. C., *J. Am. Chem. Soc.*, **50**, 1049 (1928).

little attention in a 12 hour period. Adjustments of impressed voltage for the electrolysis should be made for the best possible sensitivity and accuracy.

The measurement of O_2 consumption alone by this device is completely automatic. With the proper change of NaOH experiments have been carried on for 5 days without a cessation of the recording of O_2 consumption.

The author wishes to express his gratitude to Dr. Grinnell Jones of the Chemical Laboratory for his assistance in designing the apparatus and to Dr. W. J. Crozier for making this work possible.

SOLUBILITY OF ARAGONITE IN SALT SOLUTIONS

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(Received for publication, March 22, 1937)

Aragonite, the rhombic form of calcium carbonate, is found in the hard parts of many invertebrates and in the otoliths of some fish and amphibians. Recently it has also been found in human biliary calculi (1). It is surprising that aragonite is present in animal life, because it is usually metastable below 90°, and where solid CaCO_3 is found, one would expect it to be calcite, the stable form of calcium carbonate. Calcite, the hexagonal form of calcium carbonate, is frequently found in invertebrates. The study of why aragonite is found in some invertebrates and calcite in others, and why aragonite should be formed at all in animal life necessarily involves the question of its solubility. Bäckström (2) made a careful study of the solubility of aragonite and calcite at 9°, 25°, and 35°, at ionic strengths varying between 0.044 and 0.026. Since, however, no determinations seem to have been made at 38°, or at an ionic strength as great as that of serum, the present work was undertaken.

Theoretical

At equilibrium, and in conformity with previous usage, $[\text{Ca}^{++}] \times [\text{CO}_3^{--}] = K'_{sp}$, where K'_{sp} is the stoichiometric solubility product. In logarithmic form this equation becomes

$$-\log [\text{Ca}^{++}] - \log [\text{CO}_3^{--}] = -\log K'_{sp} = \text{p}K'_{sp}$$

It is the purpose of the present paper to present the results of the determination of $\text{p}K'_{sp}$ of aragonite as a function of ionic strength.

The $[\text{CO}_3^{--}]$ was calculated from the equation of Hastings, Murray, and Sendroy (3)

$$[\text{CO}_3]^- = \frac{[\text{total CO}_2] \times K'_1 \times K'_2}{[\text{H}^+]^2 + [\text{H}^+] \times K'_1 + K'_1 K'_2}$$

where K'_1 and K'_2 are the apparent first and second dissociation constants of carbonic acid respectively. K'_1 and K'_2 vary with the ionic strength according to the equations of Hastings and Sendroy (4), $\text{p}K'_1 = 6.33 - 0.5 \sqrt{\mu}$ and $\text{p}K'_2 = 10.22 - 1.1 \sqrt{\mu}$.

EXPERIMENTAL

Salt solutions, varying in ionic strength from 0.012 to 0.230 were equilibrated at 38° with aragonite for 20 to 40 hours. CO_2 was present in the gas phase in an amount such that the pH at equilibrium varied between 7.2 and 7.6. At the end of the equilibration period, the solutions were centrifuged and the supernatant fluid removed for analysis without exposure to air. The fluid was analyzed for its pH, CO_2 , and Ca. From these data the $[\text{CO}_3]^-$ and the product $[\text{CO}_3]^- \times [\text{Ca}^{++}]$ were calculated.

Before the experiments on the solubility of aragonite were carried out, a series of determinations of the solubility of calcite at ionic strengths varying from 0.011 to 0.228 was carried out and found to agree with those previously found by Hastings, Murray, and Sendroy (3).

pH—The pH was determined colorimetrically by having phenol red present in the solution in a concentration of 0.00075 per cent and matching the color with bicolor standards made according to the procedure of Hastings and Sendroy (5). This could be done within ± 0.01 pH. Since the ionic strength of the solution varied, it was necessary to take into account the change in the apparent dissociation constant of the indicator with ionic strength. This was done by using the appropriate constant for the particular ionic strength as determined by Sendroy and Hastings (6). The bicolor standards were checked against phosphate standards, which had been electrometrically standardized.

CO_2 —The CO_2 was determined by the method of Van Slyke and Neill (7), with the manometric gas apparatus.

Calcium—Calcium was determined according to the Clark and Collip (8) modification of the Kramer and Tisdall method.

Analyses were made in duplicate or triplicate.

TABLE I

Solubility of Aragonite At 38° in Solutions of Varying Strength

The initial composition of the solution was as follows: in Experiments 1 to 3 $\text{NaHCO}_3 = 10$ mm per liter; in all other experiments $\text{NaHCO}_3 = 25$ mm per liter. NaCl was present as stated. 40 cc. of solution were equilibrated with 0.5 gm. of aragonite.

Experiment No.	NaCl	μ	$\sqrt{\mu}$	pH	[CO ₂]	[CO ₃ "]	[Ca ⁺⁺]	pK' _{sp} CaCO ₃	
								Determined	Calculated*
	<i>m per l. × 10³</i>				<i>m per l. × 10³</i>	<i>m per l. × 10³</i>	<i>m per l. × 10³</i>		
1		0.012	0.109	7.50	13.25	0.0313	0.28	8.06	8.00
2		0.012	0.109	7.485	13.06	0.0296	0.33	8.01	8.00
3		0.012	0.109	7.58	12.92	0.0371	0.30	7.95	8.00
4		0.027	0.164	7.515	29.22	0.0821	0.19	7.81	7.82
5		0.027	0.164	7.47	30.53	0.0775	0.17	7.88	7.82
6	10	0.037	0.193	7.51	30.32	0.0907	0.18	7.79	7.74
7	20	0.047	0.218	7.47	30.15	0.0880	0.24	7.68	7.66
8	20	0.047	0.218	7.475	30.68	0.0905	0.24	7.66	7.66
9	20	0.048	0.218	7.505	30.48	0.0969	0.26	7.60	7.66
10	30	0.058	0.240	7.39	30.88	0.0778	0.31	7.62	7.61
11	40	0.067	0.260	7.455	30.13	0.0958	0.24	7.63	7.56
12	40	0.068	0.261	7.49	31.26	0.1078	0.25	7.58	7.56
13	40	0.067	0.260	7.425	29.52	0.0868	0.29	7.60	7.56
14	50	0.078	0.280	7.215	31.96	0.0588	0.57	7.47	7.51
15	70	0.098	0.312	7.495	30.04	0.1205	0.23	7.56	7.44
16	70	0.098	0.312	7.335	30.40	0.0806	0.34	7.56	7.44
17	70	0.098	0.312	7.305	30.78	0.0754	0.57	7.37	7.44
18	80	0.108	0.330	7.245	31.92	0.0715	0.57	7.39	7.40
19	90	0.118	0.344	7.365	31.24	0.0984	0.43	7.37	7.36
20	90	0.118	0.344	7.245	31.34	0.0736	0.67	7.31	7.36
21	100	0.128	0.358	7.505	30.57	0.1415	0.32	7.35	7.32
22	100	0.128	0.358	7.235	31.20	0.0749	0.68	7.30	7.32
23	110	0.138	0.372	7.345	31.18	0.1001	0.49	7.31	7.31
24	120	0.148	0.384	7.39	30.40	0.1113	0.43	7.32	7.28
25	120	0.148	0.384	7.355	30.97	0.1040	0.46	7.32	7.28
26	130	0.158	0.397	7.405	30.40	0.1184	0.41	7.32	7.25
27	130	0.159	0.399	7.225	31.50	0.0790	0.76	7.21	7.25
28	140	0.168	0.410	7.34	30.89	0.1075	0.55	7.23	7.23
29	140	0.169	0.411	7.285	30.46	0.0920	0.70	7.19	7.23
30	150	0.179	0.423	7.31	31.34	0.1031	0.71	7.14	7.21
31	160	0.189	0.434	7.26	31.40	0.0964	0.67	7.19	7.19
32	160	0.189	0.434	7.435	30.98	0.1456	0.48	7.16	7.19

* Calculated $\text{pK}'_{sp}\text{CaCO}_3$ derived from $\text{pK}'_{sp}\text{CaCO}_3 = 8.42 - \frac{4.48\sqrt{\mu}}{1 + 1.34\sqrt{\mu}}$

TABLE I—*Concluded*

Experiment No.	NaCl	μ	$\sqrt{\mu}$	pH	[CO ₂]	[CO ₃ "]	[Ca ⁺⁺]	pK' _{sp} CaCO ₃	
								Determined	Calculated*
	<i>M per l. × 10³</i>				<i>M per l. × 10³</i>	<i>M per l. × 10³</i>	<i>M per l. × 10³</i>		
33	170	0.199	0.446	7.28	31.50	0.1033	0.68	7.15	7.17
34	180	0.209	0.456	7.32	31.52	0.1169	0.60	7.15	7.15
35	180	0.209	0.456	7.30	31.72	0.1120	0.69	7.11	7.15
36	190	0.219	0.467	7.255	31.38	0.1026	0.84	7.06	7.14
37	200	0.228	0.477	7.335	30.80	0.1272	0.60	7.12	7.12
38	200	0.228	0.478	7.425	31.04	0.1605	0.44	7.15	7.12
39	200	0.229	0.478	7.315	31.12	0.1220	0.81	7.00	7.12
40	200	0.230	0.480	7.245	34.30	0.1146	0.86	7.01	7.12

Results

The analytical results and the values of pK'_{sp} calculated therefrom are given in Table I. The values of pK'_{sp} have been plotted against $\sqrt{\mu}$ in Fig. 1. The equation of the upper line, obtained as previously described (3), is

$$\text{pK}'_{sp} = 8.42 - \frac{4.48 \sqrt{\mu}}{1 + 1.34 \sqrt{\mu}}$$

where 8.42 is the pK of aragonite at zero-ionic strength.

A comparison between the values of pK' calculated from this equation and those found by experiment is given in the last two columns of Table I.

The lower line on Fig. 1 is the curve of the solubility product of calcite as a function of ionic strength. It will be seen that the pK'_{sp} of aragonite is 0.15 less than that of calcite, although the change in solubility with change in ionic strength is the same. This means that aragonite is more soluble than calcite at the same ionic strength, the ratio of the constants being $K_{\text{aragonite}}/K_{\text{calcite}} = 1.41$. When the constants are corrected to the same terms as those calculated by Bäckström (2), the agreement is satisfactory between our results and his at the same ionic strength and at the same temperature. The value of pK'_{aragonite} at the ionic strength of body fluids is 7.25, whereas that of pK'_{calcite} is 7.40.

Through the kindness of Dr. Dallas B. Phemister, we were able to determine the solubility of two gallstones which by chemical analysis consisted largely of calcium carbonate, and which, according to their x-ray spectrograms, consisted of aragonite. The pK'_{CaCO_3} values of these gallstones were respectively 7.28 and 7.34. At this ionic strength, the pK' of aragonite is 7.27, whereas that of calcite is 7.42. There is thus afforded independent evidence that

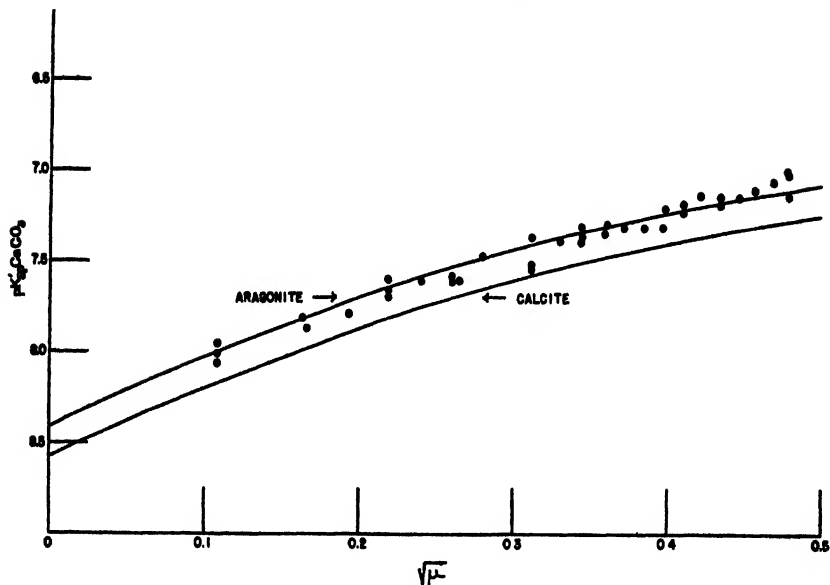


FIG. 1. The solubility of $CaCO_3$ in salt solutions of varying ionic strength saturated with $CaCO_3$ at 38° . Values of pK'_{CaCO_3} are plotted as ordinates and $\sqrt{\mu}$ as abscissæ. The lower curve represents calcite and the upper curve represents aragonite. The aragonite curve has the equation

$$pK'_{CaCO_3} = 8.42 - \frac{4.48\sqrt{\mu}}{1 + 1.34\sqrt{\mu}}$$

the calcium carbonate of these gallstones belonged to the rhombic form aragonite.

SUMMARY

The solubility of aragonite has been studied as a function of ionic strength and compared with that of calcite.

Aragonite is more soluble than calcite, the ratio of their solubility products being 1.41.

The solubility product of calcium carbonate gallstones has been found to correspond to that of aragonite.

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STUDIES IN AMINO ACID METABOLISM

II. FATE OF *d*-GLUTAMIC, *dl*-GLUTAMIC, *dl*-PYROGLUTAMIC, *l*-ASPARTIC, AND *dl*-ASPARTIC ACIDS IN THE NORMAL ANIMAL*

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It has been shown earlier (1) that *d*-alanine was approximately twice as good a glycogenic agent as *dl*-alanine, while the latter was far superior to glycine in this respect. Moreover, their ketolytic activity showed a similar order of magnitude. Such results are difficult to interpret in the light of the classical experiments of Ringer and Lusk (2) in which it was demonstrated that these amino acids are converted to glucose to the extent of 100 per cent in phlorhizinized dogs. These discrepancies with the phlorhizin technique make an evaluation of the glycogenic and ketolytic activity of the other amino acids desirable.

Lusk (3) concluded that *d*-glutamic acid was three-fifths converted to glucose and that when (2) *l*-aspartic acid was fed sugar accounting for only 3 of the 4 carbon atoms was excreted by phlorhizinized dogs. Using as a criterion the deposition of glycogen in the liver and muscles of rats after feeding the amino acid, Wilson and Lewis (4) have concluded that *d*-alanine, *dl*-alanine, and *d*-glutamic acid are glycogen formers, while *l*-leucine and glycine give negative results. Reid (5) reported that glutamic and aspartic acids failed to cause any increase in the liver glycogen of anesthetized cats.

* This work was made possible through a grant from the Rockefeller Foundation.

A preliminary report of this work was given before the American Society of Biological Chemists at Memphis, April 21-24, 1937.

Several papers have appeared which have shown uniformly that pyroglutamic acid at least can undergo metabolism. Abderhalden and Hanslian (6) reported that there was a difference in the metabolism of the two isomers. In rabbits apparently the *l* form was more readily broken down, while man metabolized the *d* form more easily. Bethke and Steenbock (7) concluded that pigs were able to break the ring when *l*-pyroglutamic acid was fed. This conclusion is based upon change in nitrogen excretion. Greenberg and Schmidt (8) using dogs came to essentially the same conclusions.

EXPERIMENTAL

For the glycogen studies male rats from our own colony, ranging from 150 to 225 gm., were used. With the exception of the pyroglutamic acid, which was fed as the free acid, all amino acids were given as the monosodium salts. The material was administered by stomach tube hourly, so that there was always a greater amount of the amino acid present in the gastrointestinal tract than could be absorbed during the hour. Consequently maximum absorption was occurring at all times. This is the method used by Wilson and Lewis (4).

The absorption was allowed to continue for 2, 4, 6, 8, 10, or 12 hours, at which time the animal was killed and the liver used for the determination of glycogen. At the same time the gastrointestinal tract was removed and the contents washed out. These were precipitated with trichloroacetic acid and the amino nitrogen was determined on the filtrate. The method of Good, Kramer, and Somogyi (9) was employed for the estimation of glycogen. In some of the early experiments the animals developed diarrhea and it was noted that if fecal material was present in the gastrointestinal tract no diarrhea developed. Accordingly in the later tests the rats were allowed to eat filter paper which gave enough bulk so that not more than 3 per cent of the animals developed diarrhea, where formerly 70 per cent of the experiments had been unsuccessful for this reason.

For confirmatory evidence of the metabolic fate of these compounds their ketolytic activity was studied by the method of Butts and Deuel (10). This consisted of feeding sodium butyrate in larger amounts than the animal could burn and then superimposing upon this ketonuria the material in question. The con-

trols received an equivalent amount of sodium chloride solution. Urine collections were made every 24 hours.

The analyses for total acetone bodies were carried out by the Van Slyke procedure and those for total nitrogen by the Kjeldahl method. Female rats were used for this part of the work. Because the 1st day seemed to produce very irregular results, the animals received only the sodium butyrate on this day and no analyses were made. Furthermore, the animals were not subjected to a preliminary overnight fast.

Results

In a control series of twenty-five male animals, fasted 2 days but receiving filter paper, the mean for liver glycogen was 0.35 per cent. The values ranged from 0.03 to 0.68 per cent. This level agrees very closely with that for a group of males reported from this laboratory which had been fasted the same period but which had not received the filter paper (11).

In Table I are given the results on liver glycogen after feeding the monosodium salts of *d*-glutamic, *dl*-glutamic, *l*-aspartic, and *dl*-aspartic acids and *dl*-pyroglutamic acid as the free acid. Too many experiments were performed to give individual values but averages are reported and for certain periods the mean difference divided by the probable error of the mean difference is given. If this value is greater than 3, the differences are considered significant. A comparison between the groups receiving the amino acids and the control animals receiving only water shows a decided significance for all of the longer periods and in most cases the shorter ones, even though many of the groups contain only five to seven animals. The actual values of the liver glycogen for the 12 hour periods are as follows: *d*-glutamic acid 1.25 per cent, *dl*-glutamic acid 0.98 per cent, *l*-aspartic acid 2.31 per cent, *dl*-aspartic acid 1.95 per cent.

The ketolytic effect of the amino acids is summarized in Tables II and III.

Named in the order of their efficiency in regard to this property one finds *dl*-alanine best, followed by *dl*-pyroglutamic acid, *l*-aspartic acid, *d*-glutamic acid, and *dl*-aspartic acid. *dl*-Alanine was included in this study as a means of comparison, since previous work had established it as an excellent ketolytic substance (1).

It occurred to us that perhaps the presence of filter paper in the gastrointestinal tract of the rats might lead to the production of

TABLE I

Glycogen Determinations on Livers of Male Rats after Receiving Various Amino Acids at Varying Intervals

Control glycogen, 0.35 per cent (25 experiments).

Material fed	Per cent glycogen after maximum absorption					
	2 hrs.	4 hrs.	6 hrs.	8 hrs.	10 hrs.	12 hrs.
<i>d</i> -Glutamic acid	0.19 (6)	0.50 (7) 2.78	0.73 (7) 4.74	0.78 (5) 2.51	0.88 (6) 7.24	1.25 (6) 5.47
<i>dl</i> -Glutamic acid	0.32 (7)	0.27 (6)	0.66 (6) 3.32	0.56 (6) 2.63		0.98 (9) 4.96
<i>dl</i> -Pyroglutamic acid					0.94 (4) 11.20	
<i>l</i> -Aspartic acid	0.64 (8) 4.26	1.02 (7) 7.44	1.04 (12) 11.60	1.81 (7) 14.90	1.55 (12) 9.33	2.31 (7) 8.08
<i>dl</i> -Aspartic acid	0.34 (7)	0.61 (6) 2.94	0.89 (6) 5.48	1.20 (6) 8.57	1.52 (7) 5.66	1.95 (6) 7.60

The figures in parentheses are the number of experiments in the averages.

The figures in bold-faced type represent M.D./P.M. (M.D.) in comparison to control level.

TABLE II

*Acetone Body Excretion of Female Rats Receiving Sodium Butyrate Plus Either *dl*-Alanine, *dl*-Pyroglutamic Acid, *d*-Glutamic Acid, *dl*-Aspartic Acid, *l*-Aspartic Acid, or an Equivalent Amount of NaCl Solution*

Material fed	Amount fed	Total acetone body excretion daily		
		1st day	2nd day	3rd day
	gm. per sq. m.	gm. per sq. m.	gm. per sq. m.	gm. per sq. m.
Control.....		1.22 (14)	3.80 (13)	5.07 (12)
<i>dl</i> -Alanine.....	10.70	0.66 (8)	0.96 (8)	0.54 (8)
<i>d</i> -Glutamic acid.....	17.40	0.58 (8)	1.46 (7)	1.60 (6)
<i>dl</i> -Pyroglutamic acid.....	15.25	0.62 (8)	1.08 (7)	1.01 (6)
<i>l</i> -Aspartic acid.....	16.60	0.61 (14)	1.33 (13)	1.22 (6)
<i>dl</i> -Aspartic acid.....	16.60	0.87 (16)	2.27 (12)	2.93 (7)

The values in parentheses are the number of experiments in the averages.

some sugar-forming metabolite with consequent increase of liver glycogen. The fact that the controls receiving filter paper were

at a level comparable with a group which did not ingest paper seems to answer this criticism. As a further check a bacteriological study was carried out in an attempt to determine whether or not cellulose-splitting organisms were present in the intestine of the rats from our own colony.¹ It is known that rats under certain environmental conditions may have such bacteria in their large intestines. The animals were fasted 3 days, during which time they were allowed paper *ad libitum*. They were then killed and the gut washed with 50 cc. of saline. The resulting suspension

TABLE III

Average Acetone Body Excretion of Female Rats after Receiving Sodium Butyrate Plus Either Isomolecular Amounts of dl-Alanine, dl-Pyrogutamic Acid, d-Glutamic Acid, l-Aspartic Acid, or dl-Aspartic Acid, or an Equivalent Amount of NaCl Solution

Substance fed	No. of experiments	Acetone bodies			P.M., mean	M.D./P.M. (M.D.) compared with				
		Minimum	Maximum	Mean		I	II	III	IV	V
		gm. per sq. m.	gm. per sq. m.	gm. per sq. m.						
Control (I).....	39	0.25	9.43	3.29	0.259					
d-Glutamic acid (II)...	21	0.19	3.06	1.16	0.128	7.37				
dl-Pyrogutamic acid (III).....	21	0.07	2.56	0.95	0.122	8.18	1.09			
l-Aspartic acid (IV)...	33	0.04	3.13	1.01	0.147	7.65	0.76	0.31		
dl-Aspartic acid (V)...	35	0.00	5.13	1.76	0.285	4.04	1.91	2.60	2.33	
dl-Alanine (VI).....	24	0.00	2.04	0.72	0.072	9.63	3.07	1.63	1.77	3.56

was shaken well and coarse material was allowed to settle. 1 cc. of the liquid was inoculated into each tube of the following media: (1) nutrient broth, (2) cellulose medium without cellulose, (3) nutrient broth and cellulose, (4) cellulose medium and cellulose, (5) cellulose medium plus cellulose with 0.5 per cent *d*-glutamic acid, and (6) broth plus cellulose plus 0.5 per cent *d*-glutamic acid. In each case 1 gm. of filter paper was used. The tubes were sealed with paraffin for anaerobic culture and incubated at 37° and 55°

¹ The authors wish to express their appreciation to Mr. Ralph Tracy of the Department of Bacteriology for the bacteriological studies.

for 2 weeks. A similar test was made with fecal material from animals receiving cellulose. 0.1 to 0.5 gm. were inoculated into the same sets of tubes as described above and incubated 3 weeks. These tubes were not sealed. Without exception cellulose-splitting bacteria were absent as judged by cellulose utilization, although growth of other organisms occurred in the broth.

DISCUSSION

Although Wilson and Lewis (4) report that *d*-glutamic acid is a glycogen former, their values even for the longer periods do not approach the values which we obtained. We were unsuccessful in studying this problem until bulk was supplied in the form of filter paper, because a great majority of our animals developed diarrhea, and even those which did not showed irritated gastrointestinal tracts and low glycogen levels. Thereafter this difficulty was overcome and the experiment successfully terminated. Apparently the speed of absorption was not interfered with, because the rates obtained in our experiments correspond closely with the figures given by Wilson and Lewis for *d*-glutamic acid.

The actual levels for liver glycogen after *d*-glutamic acid are as follows for 2, 4, 6, 8, 10, and 12 hours respectively: 0.19, 0.50, 0.73, 0.78, 0.88, and 1.25 per cent. For *dl*-glutamic acid somewhat lower values were obtained. No 10 hour group is reported but values for the other periods are as follows: 0.32, 0.27, 0.66, 0.56, and 0.98 per cent. The fact that the racemic mixture does not form glycogen as readily as does the natural (*d*) isomer is in accord with earlier work reported with *d*- and *dl*-alanine (1). The differences are not as accentuated, because as a glycogenic agent glutamic acid cannot compare with alanine. With the exception of the 2, 4, and 8 hour groups the differences in glycogen level are statistically significant. The differences between the *d* and *dl* mixtures are not statistically significant but in every case the levels are different and presumably if enough experiments were carried out a significant value could be reached.

The *dl*-pyroglutamic acid which was fed as the free acid also gave rise to a considerable quantity of glycogen. Although very few experiments on only one period are reported, apparently the body can break the pyrrole ring and metabolize this compound.

Statistically the value is shown to be valid, and as a glycogenic agent it compares favorably with glutamic acid.

The *l*- and *dl*-aspartic acids fed as the monosodium salts also were found to be quite good glycogen formers. The values as reported in Table I show that the natural (*l*) isomer is somewhat better in this respect than the *dl* mixture. Without exception the *l* isomer gave significantly higher results than were observed in the fasting controls with the following values of liver glycogen for the 2, 4, 6, 8, 10, and 12 hour groups, respectively: 0.64, 1.02, 1.04, 1.81, 1.55, and 2.31 per cent. The values for *dl*-aspartic acid for similar periods were 0.34, 0.61, 0.89, 1.20, 1.52, and 1.95 per cent. With the exception of the 2 and 4 hour periods all of the latter results are significant when compared with the control level of liver glycogen. Although the differences between the averages of glycogen after the *l* and the *dl* mixtures are not statistically significant because of the limited number of tests, one does find the values of liver glycogen given by the natural isomer consistently higher than those following the *dl* compound.

In the experiments on ketonuria the control levels of total acetone bodies when no amino acid was given along with the sodium butyrate were for the 1st, 2nd, and 3rd days expressed in gm. per sq.m. as follows: 1.22, 3.80, and 5.07. After *dl*-alanine, which has been demonstrated to be highly ketolytic, the lowest values were recorded. These were 0.66, 0.96, and 0.54 for the 3 days. The averages after feeding isomolecular amounts of *d*-glutamic acid, *dl*-pyroglutamic acid, and *l*-aspartic acid along with the sodium butyrate were all close to the same value but the acetone body excretion after giving *dl*-aspartic acid was definitely higher than that recorded after feeding any of the other compounds. Compared to the controls there was a significant difference in all cases. Such an evaluation is given in Table III. The ketolytic activity is further evidence of the fact that these amino acids are glycogenic if we accept the results of Shapiro (12).

There can be no doubt that these amino acids follow the pathway in metabolism shown by phlorhizin experiments; namely, that they are all sugar formers. The results of the ketolytic studies also show this is to be true, but they do not necessarily follow the glycogen studies quantitatively. For instance, *dl*-

aspartic acid is more effective when maximum absorption occurs as a glycogen former than is *d*-glutamic acid but the reverse is true when isomolecular amounts are fed along with sodium butyrate. This may mean that (a) *dl*-aspartic acid is more readily absorbed than *d*-glutamic acid when large amounts are fed, but the small amount given in the ketosis experiments is within the range which would be absorbed readily in both cases, or (b) the tolerance for *d*-glutamic acid is less than *dl*-aspartic and a considerable amount of that which is being absorbed is escaping metabolism by excretion in the urine, or (c) a compound can show ketolytic properties without forming glycogen. Of the three possibilities the second postulate seems to be the most logical.

The fact that the same metabolic differences between the natural isomers and the racemic mixtures hold for glutamic and aspartic acids as were reported for the alanines lends strength to the hypothesis that one form is more important in metabolism than is the other. In each case studied the natural isomer has appeared to be the effective form.

SUMMARY

1. *d*-Glutamic acid has been shown to be definitely glycogenic and to possess ketolytic properties. As a glycogen former it is somewhat superior to *dl*-glutamic acid.

2. *dl*-Aspartic acid is much superior to *d*-glutamic acid as a glycogen former during maximum absorption but when isomolecular amounts are fed it is distinctly inferior to the natural isomer of glutamic acid as a ketolytic agent.

3. *l*-Aspartic acid is very effective as a glycogen former when maximum absorption is allowed and compares favorably with *d*-glutamic acid as a ketolytic agent. The natural isomer is definitely better in both respects than *dl*-aspartic acid.

4. *dl*-Pyroglutamic acid undergoes metabolism and apparently follows, both quantitatively and qualitatively, the same pathway as glutamic acid.

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STUDIES ON KETOSIS

XI. THE RELATION OF FATTY LIVERS TO FASTING KETONURIA IN THE RAT*

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There can be no question but that fat is the chief source of the ketone bodies which are excreted in the urine under a variety of circumstances. In the rat, however, no appreciable ketonuria occurs during fasting, although large quantities of fat are present in the carcass and must represent the principal source of energy during the period of inanition. Moreover, the administration of comparatively large amounts of such neutral fat as tributyrin or tricaproin is not followed by a marked ketonuria (1) in such animals, although, when the sodium salts or the ethyl esters of the even chain fatty acids are fed, a considerable ketonuria results (2, 3).

The fat present in the liver above the amount which may be considered the *élément constant* of Terroine must represent the chief source of ketone bodies during fasting. That this is true seems especially evident when one realizes that the liver is the principal site of ketone body synthesis (4). In the rat, however, no appreciable increase in lipid content follows inanition; this phenomenon probably explains the absence of well defined fasting ketosis in this species (5).

There have been no extensive studies designed to ascertain whether a correlation exists between high liver fat and ketonuria

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in the rat. The present study was made on rats in which the fatty livers were produced by high fat, high carbohydrate, low protein diets similar to those employed by Best and Channon (6). It was also of interest to determine whether the sex differ-

TABLE I

Liver Glycogen, Water, and Lipids in Unfasted Male and Female Rats for 14 Days on Various High Fat Diets (Group A)

Previous diet	No. of experiments		Liver, per cent of body weight		Liver water		Liver glycogen		Liver lipids	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
					per cent	per cent	per cent	per cent	per cent	per cent
Stock diet.....	15	16	4.11	4.11	67.5	68.4	4.28	2.29	3.76	3.79
Butter Fat I†....	10	10	3.84	4.76	54.0	54.2	8.53*	1.93	23.07	27.28
“ “ II....	15	20	4.86	5.34	50.0	48.6	3.45	1.51	32.35	35.26
Cod liver oil....	14	15	4.18	3.82	64.9	67.8	3.92	3.36	11.80	7.28
Coconut oil.....	15	15	5.34	5.32	52.9	53.1	5.08	2.71	28.42	28.82
Cholesterol-Lard I.....	15	15	6.20	6.68	49.4	47.3	3.70	1.43	34.92	37.92
Cholesterol-Lard II†.....	5	5	4.56	4.30	57.8	60.4	2.75	1.67	18.21	16.17

* Ratio of mean difference to probable error of mean difference of the average of males compared with the mean of females. When this value exceeds 3.00, the differences are considered significant.

† No yeast used in this series. Crystalline vitamin B₁, kindly supplied by Merck and Company, given daily by stomach tube in a dose of 0.01 mg. daily.

‡ High protein diet (20 per cent casein) at expense of glucose.

ences in ketonuria noted earlier (2) are related to such differences in liver lipid; and finally a comparison has been made in the ketonuria resulting after fatty livers had been produced by the administration of various fats.

General Procedure

Male and female rats, 3 to 4 months old, from our stock colony and previously on our stock diet (3) were used. They were placed in separate cages and fed on the diet under investigation for 14

TABLE II

Water and Lipids in Male and Female Rats Fasted 3 Days Following 14 Days on Various High Fat Diets (Group B)

Previous diet	No. of experiments		Body weight		Liver, per cent of body weight		Liver water		Liver lipids	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
			gm.	gm.			per cent	per cent	per cent	per cent
Stock diet.....	10	10	164	120	3.23	3.53	68.4	69.5	3.95	3.61
			-29	-24	-0.88	-0.58	+0.9	+1.1	+0.19	-0.18
Butter Fat I*..	10	10	155	111	3.74	4.07	54.2	59.6	23.76	19.99
			-24	-18	-0.10	-0.69	+0.2	+5.4	+0.69	-7.29
“ “ II..	10	9	177	133	4.27	5.19	52.2	51.9	30.59	30.38
			-10	-24	-0.59	-0.15	+2.2	+3.3	-1.76	-4.88
Cod liver oil....	10	10	127	102	3.33	3.36	67.2	70.2	8.44	4.22
			-14	-16	-0.80	-0.46	+2.3	+2.4	-3.26	-3.04
Coconut oil....	10	10	128	104	4.53	4.20	55.8	61.5	25.49	17.28
			-25	-17	-0.79	-1.12	+2.1	+8.0	-1.30	-10.45
Cholesterol-Lard I.....	10	10	172	117	5.40	6.24	47.6	47.3	36.44	37.64
					-0.60	-0.44	-1.8	0.0	+1.52	-0.28
Cholesterol-Lard II†.....	5	5	196	127	3.82	4.13	55.4	57.8	23.90	18.10
			-24	-22	-0.74	-0.17	-2.4	+0.5	+5.49	+1.93

The values in bold-faced type represent the change in mean when compared with the averages for unfasted rats (Group A, Table I). The loss in body weight in the present series is for rats fasting for 3 days.

* No yeast used in this series. Crystalline vitamin B₁, kindly supplied by Merck and Company, given daily by stomach tube in a dose of 0.01 mg.

† High protein diet (20 per cent casein) at expense of glucose.

days. At the conclusion of this period they were divided into two groups. The first, Group A, was sacrificed immediately; glycogen, water, fat, and protein¹ were determined in the liver of the unfasted

¹ The results on liver protein will be reported in a later communication.

rats which consisted of an equal number of each sex. Group B, which was composed of litter mates of the animals used in Group A, was fasted for 3 days, during which the urine was collected by the procedure usually employed in our studies on ketonuria. Nitrogen and acetone bodies were determined on the urine by the Kjeldahl procedure and the Van Slyke technique respectively. Sodium chloride solution was administered by stomach tube twice

TABLE III
*Water and Lipids in Livers of Male and Female Rats Fasted 5 Days
Following 14 Days on Various High Fat Diets (Group C)*

Previous diet	No. of experiments		Body weight		Liver, per cent of body weight		Liver water		Liver lipids	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
			gm.	gm.			per cent	per cent	per cent	per cent
Stock diet....	5	5	169	118	2.80	3.49	70.1	72.2	4.31	4.07
			-40	-30	-1.31	-0.62	+2.6	+3.8	+0.55	+0.28
Butter Fat II.	5	5	163	115	4.95	4.41	53.7	58.4	31.68	22.38
			-22	-24	+0.09	-0.93	+3.7	+9.8	-0.67	-12.50
Cod liver oil..	4	5	163	114	3.13	3.30	66.0	70.7	12.09	5.94
			-22	-20	-1.00	-0.52	+1.1	+2.9	+0.29	-1.32
Coconut oil ..	15	13	163	124	3.35	4.02	60.8	63.6	19.85	16.85*
			-27	-19	-1.99	-1.30	+7.9	+10.5	-8.57	-12.17
Cholesterol- Lard I.....	5	5	143	115	5.04	5.23	50.1	50.4	34.32	34.36
			-23	-24	-1.16	-1.45	+0.7	+3.1	-0.60	-3.56

The values in bold-faced type represent the change in mean when compared with the averages of the corresponding values of unfasted rats (Group A, Table I). The loss in body weight is for the rats in the present series on fasting for 5 days.

* Two abnormally high results were excluded from the average.

daily (1 cc. per 100 sq.cm. of surface area) to produce a diuresis. At the conclusion of the experiments on ketonuria, the animals were sacrificed and the livers removed to determine the lipid, water, and protein¹ contents. In Group C the ketosis tests were continued for 5 days, after which the animals were killed and similar analyses made. Amytal was employed throughout as an anesthetic when the animals were sacrificed.

The glycogen was determined by the method of Good, Kramer, and Somogyi (7). Liver water was ascertained by drying the liver to a constant weight in a vacuum oven heated to 50°. The determination of liver lipid was made on the dried sample by extraction with ether on the Bailey-Walker apparatus, followed by a re-extraction of the liver which was powdered after the first extraction.

TABLE IV
Average Body Weight and Food Consumption of Rats on Various High Fat Diets

Diet	Body weight at start of diet						Food consumption per 100 gm. per day					
	Males			Females *			Males			Females		
	Group A	Group B	Group C	Group A	Group B	Group C	Group A	Group B	Group C	Group A	Group B	Group C
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Butter Fat I	208	210		143	149		5.18	4.02		4.71	7.16	
“ “ II	-33	-31		-16	-20							
	200	203	207	152	170	151	4.69	4.46	4.59	4.92	4.16	4.48
	-6	-16	-22	-8	-13	-12						
Cod liver oil	190	180	221	149	140	153	3.89	3.38	3.03	4.56	4.62	4.07
	-31	-39	-36	-25	-22	-32						
Coconut oil	191	172	203	160	140	158	5.06	7.39*	3.64	6.12*	8.98*	3.87
	-27	-30	-13	-20	-19	-13						
Cholesterol-	192	205	188	139	141	148	4.66	3.97	4.56	5.39	4.12	4.34
Lard I	-19	-22	-22	-9	-14	-9						
Cholesterol-	198	210		148	142		4.18	4.50		5.07	4.95	
Lard II	+5	+17		+10	+7							

The values under the average body weight represent the mean change in body weight during the 14 day period when the diet was ingested. The number of experiments in each group is the same as in the experiments recorded in Tables I to III.

* Maximum values; a considerable amount was spilled from the cups.

In general the diets had the following composition, which is similar to those employed by Channon and coworkers: casein² 5 per cent, glucose (cerelose) 48 per cent, fat under investigation 40 per cent, irradiated yeast³ 2 per cent, salt mixture (8) 5 per cent.

² Vitamin-free, from the Casein Manufacturing Company of America.

³ Specially high in vitamin G, furnished by Standard Brands, Inc.

TABLE V

Acetone Bodies in Urine of Fasting Male and Female Rats Previously on High Fat Diets, Receiving Sodium Chloride Solution

Previous diet	Acetone bodies, gm. per sq. m.										Acetonuria for 100 gm. rat ^a	
	1st day		2nd day		3rd day		4th day		5th day		Male	Female
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female		
Stock diet (controls)	0.04 (15)	0.04 (15)	0.03 (15)	0.01 (15)	0.05 (15)	0.10 (15)	0.17 (5)	0.13 (5)	0.30 (5)	0.35 (5)	0.8	1.1
Butter Fat I†	0.03 (10)	0.38 (10)	1.10 (10)	3.29 (10)	1.88 (10)	2.43 (10)					24.7	57.0
	3.34		5.96		3.10							
Butter Fat II	0.06 (14)	0.53 (13)	1.80 (15)	2.73 (14)	1.88 (15)	2.95 (14)	1.46 (4)	2.16 (5)	0.91 (4)	1.83 (5)	36.6	56.5
	3.40		2.82		3.64		2.87					
Cod liver oil	0.15 (15)	0.31 (14)	2.99 (14)	4.12 (14)	1.87 (15)	3.05 (15)	1.77 (5)	3.06 (5)	1.05 (5)	2.64 (5)	48.3	71.2
	1.71		2.86		3.87		3.81					
Coconut oil	0.25 (15)	0.41 (14)	2.83 (15)	3.03 (15)	1.84 (15)	2.15 (15)	1.65 (5)	2.41 (5)	0.73 (5)	1.68 (5)	46.5	51.6
							2.74					
Cholesterol-Lard I	0.12 (14)	0.51 (14)	2.44 (15)	3.10 (13)	2.79 (15)	3.00 (12)	1.57 (5)	1.16 (5)	0.82 (5)	0.62 (5)	52.2	60.8
	4.38		2.27		0.81							
Cholesterol-Lard II	0.00 (5)	0.00 (5)	0.17 (5)	0.23 (5)	0.33 (5)	1.46 (5)					5.0	16.7

The figures in parentheses are the number of experiments included in the average.

The figures in bold-faced type represent the ratio of mean difference to probable error of mean difference for the average of males compared with the mean of the same group of females. The ratio for the 4th day is based on the combined average of the 4th and 5th days respectively.

* Average for 2nd and 3rd days only.

† No yeast used in this series. Crystalline vitamin B₁, kindly supplied by Merck and Company, given daily by stomach tube in a dose of 0.01 mg.

In the experiments with cholesterol (Eastman) lard was employed to make the food cohere. This comprised 40 per cent of the diet. 2 per cent of cholesterol was added at the expense of the cerelese.

Results

The summary of the analyses on Group A of the glycogen, fat, and water in the livers of control rats (on the stock diet) and of those previously on the special high fat diet is presented in Table I. Table II gives the results on Group B, while Table III records the mean values for Group C. In Table IV are shown data as to food consumption, etc., for the animals whose experiments are recorded in the other protocols.

The studies on ketonuria made on rats previously on the various high fat diets are recorded in Table V. The analyses of the livers

TABLE VI.

Average Nitrogen, in Gm. per Sq. M., in Urine of Fasting Male and Female Rats Following Various Diets

Previous diet	Males					Females				
	1st day	2nd day	3rd day	4th day	5th day	1st day	2nd day	3rd day	4th day	5th day
Stock Diet	4.19	4.41	3.51	3.28	2.86	4.52	4.37	3.76	3.25	3.10
Butter Fat I.	2.42	2.78	2.84			2.67	3.27	3.30		
" " II.	2.55	2.87	3.01	3.11	2.79	2.57	3.13	2.71	3.08	2.86
Cod liver oil.	2.81	3.23	2.95	2.71	2.52	2.68	3.13	2.85	2.52	2.57
Coconut oil.	2.89	3.05	3.05	2.57	2.19	2.67	3.03	2.97	2.48	2.10
Cholesterol-Lard										
I.	2.43	3.10	3.05	3.03	3.18	2.75	3.05	3.04	3.10	2.89
Cholesterol-Lard										
II.	4.58	3.64	3.24			4.31	3.71	3.35		

The values are averages of a similar number of experiments in each case as those reported in Table V.

of the animals used in these tests following the 3 day fast are recorded in Table II, while the values for those undergoing the 5 day tests are given in Table III. The values for urine nitrogen are given in Table VI.

DISCUSSION

The extent to which a ketonuria develops during fasting in the rat is principally related to the nature of the previous diet. In rats previously receiving our stock diet (which contains 5.40 per cent of fat), there was no appreciable ketonuria during a 5 day period of fasting. Such results are in harmony with our earlier

work (2), although it was found that the incorporation of 5 per cent of desiccated liver in such a diet was followed by the appearance of definite amounts of the acetone bodies during inanition (9). The fasting ketonuria in these rats on the liver diet could be abolished by returning them for 60 days to a similar liver-free régime.

On the other hand a considerable ketonuria which reaches a maximum on the 2nd or 3rd fast day occurs when the rats have been on a high fat diet of such a nature that a deposition of liver fat has resulted. Such a condition persists for at least 5 days, although the quantity of acetone bodies is considerably reduced from the maximum level by that time. In all the experiments recorded here the average value for the excretion of ketone bodies is higher with the females. In most instances the variations are statistically significant. Although such a sex difference in fasting ketonuria has previously been noted between normal men and women (10) as well as in fasting rats and guinea pigs fed sodium acetoacetate (2), this is the first instance in which such a sex variation has been found in rats when the material is not derived from exogenous sources.

The extent of the ketonuria is not definitely related to the fat level but may possibly be altered by the type of liver fat laid down. Thus, a higher level of ketonuria was noted in the animals previously receiving the cod liver oil than in those on the butter fat diet; the liver fat was 3 to 5 times as great in the butter fat group as was found in the animals fed cod liver oil (butter fat, males 32.35 per cent, females 35.26 per cent; cod liver oil, males 11.80 per cent, females 7.26 per cent). Moreover, although the level of ketonuria was invariably higher in the female rats, the greater level cannot be traced to the liver fat. It is true that in the majority of experiments the liver fat is somewhat higher in the female group. However, owing to considerable variations in the individual experiments such differences are not statistically significant. It is of interest to note that Campbell (11) has also reported that the fat deposition in the livers of female guinea pigs receiving anterior pituitary extracts was greater than in the male animals. In the group of experiments with cod liver oil, in which much higher liver fat was noted in the males, the female animals still excreted significantly greater amounts of the acetone bodies.

That a qualitative difference according to sex exists in the type of fat present in the livers seems to be suggested by the results obtained following a 3 or 5 day fast. That the lipids in this organ of the female are much more labile than those of the male is indicated by the constant results on the decrease in liver fat in Groups B and C (recorded in Tables II and III). In all cases the fall in liver fat from the level in unfasted animals is much greater in the females. Thus, on Butter Fat I there is a decline of 7.69 per cent in the female group after 3 days of fasting, while there is an increase of 0.69 per cent in the males. In the coconut oil tests, the drop for a 3 day fast is 10.45 per cent in the females as contrasted with a fall of only 1.30 per cent in the males. In those experiments on Cholesterol-Lard II in which an increase in liver fat results after 3 days in both the males and females, the rise is considerably less in the latter group. In the animals on Butter Fat II, a decrease in 12.50 per cent was noted after a 5 day fast, while there was very little change in the case of the male animals (-0.67 per cent). The only divergent group in which the decrease in fat in the females was less than in the males was in the 3 day tests on cod liver oil. However, the level in the females had dropped to the value of 4.22 per cent and this is approximately the level of the *élément constant* of Terroine. It approximates the value found in the animals on the stock diet (3.79 per cent). The values noted in this experiment also probably would have been in harmony with the rest had not the fat level of the female livers approached what one might consider as an "irreducible minimum" of liver fat.

The rate of disappearance of liver fat is definitely the slowest in the rats which have been fed on a diet high in cholesterol. After a 3 day fast the fat in the liver of the males had increased 5.49 per cent (Cholesterol-Lard II), while that in the females was 1.93 per cent higher.

The levels of liver fat are somewhat higher than those reported by Channon and Wilkinson (12). These investigators give mean figures of 30.67 per cent for the butter fat, 20.54 per cent for the coconut oil, and 7.18 per cent for the cod liver oil tests. The reason why the cod liver oil diet fails to bring about a better fat deposition in the liver is obscure. Higher values are noted in some cases in individual experiments. A liver fat as high as 22.94 per cent was obtained in one of our tests on male rats. It is

probable that the low values may largely be ascribed to the low food intake of the rats receiving the cod liver oil diet, as suggested by Channon and Wilkinson.

A consistent sex difference in liver glycogen of the unfasted rats was noted in the present experiments. In all cases except the tests with coconut oil, the liver glycogen of the male rats was significantly higher than that of the females. The mean value of liver glycogen in the females after Butter Fat II was only 49.5 per cent of that found in the males, while a maximum of 73.4 per cent of the level found in males was obtained in the female rats previously fed coconut oil. In earlier work from this laboratory (5) we have noted that female rats have lower stores of glycogen in the liver than males for periods of fasting up to 4 days. The sex difference in glycogen level of fasted rats has been confirmed by Blatherwick and coworkers (13). Although no differences were noted in *unfasted* animals on the stock diet in the earlier work, the present experiments leave no doubt that such a divergence does occur, not only after fat diets but after an essentially similar stock diet. We are unable to explain the failure to note the sex difference in unfasted animals in our earlier report; however, the animals used in our previous work were obtained from commercial sources and they were not so uniform or in as good nutritional condition as those used in the present tests in which all came from our own colony.

The liver water is inversely proportional to the fat content. While it averages about 70 per cent in the animals on the stock diet (with a fat content of 3.76 per cent), a mean value as low as 47.3 per cent was noted in the cholesterol tests (37.92 per cent of fat). After intervals of fasting of 3 or 5 days, the rise of percentage in liver water is invariably higher in the females than the males. This fact offers further support to the more rapid decrease in liver fat in the former case. The percentage of the total body weight attributable to the liver is usually higher in the fat-fed animals. In the tests on Cholesterol I in which the maximum liver fat was noted, the average value is 6.68 per cent compared with a normal percentage of 4.11.

The lipotrophic action of casein is illustrated in the tests on Cholesterol-Lard II. This diet contained 20 per cent of casein instead of the usual 5 per cent. The level of the liver fat was

reduced to approximately 50 per cent of that noted after a similar fat was fed with the low protein level. A slight ketonuria developed during fasting after this diet, although it was the lowest level of any except the control group.

The loss in weight noted in all groups of tests (except Cholesterol-Lard II) during the 14 day period when the diets were ingested is probably traceable to an insufficient protein intake. Although the food intake of those rats on the cholesterol diet which contained 20 per cent of casein (Cholesterol-Lard II) is no greater than that of the rats on the other diet, the former gained 5 to 17 gm. on an average compared with an average loss of 6 to 33 gm. in the other tests. The protein lack is also evident in the average value of urinary nitrogen. In the present tests the values are usually 3.00 gm. per sq.m. or less, while with the control rats previously on the stock diet the averages per day for the 5 day fast were 4.19, 4.41, 3.51, 3.28, and 2.86 gm. respectively. Similarly, the urinary nitrogen for Cholesterol-Lard II was 4.58, 3.64, and 3.24 gm. for the 3 day period. It should be noted that the high ketonuria may be related to the low endogenous protein metabolism (as reported by Shapiro (14)) as well as to the high liver fat.

The rats ate about 4 gm. of food per day per 100 gm. rat. In spite of the fact that this is only about one-half the level in Channon's tests, the levels of liver fat which we obtained are considerably higher.

SUMMARY

A fasting ketonuria followed the administration to rats of diets high in butter fat, coconut oil, cod liver oil, and cholesterol-lard although no appreciable excretion of acetone bodies occurred during a 5 day period following a low fat diet. There was no direct relationship between the height of liver fat and the magnitude of the ketonuria. In the experiments on cod liver oil in which the liver fat was lowest, the ketonuria reached the maximum level.

Female rats consistently have a higher level of ketonuria than the males. It does not seem probable that this is associated to any degree with the quantity of liver fat available at the start of the fast, since a similar difference was noted in the cod liver oil

tests in which the liver fat was higher in the male rats. It seems quite likely that it is to be traced to a qualitative difference of the liver fat. The fat in the female apparently is much more labile than that in the male, as noted by the consistently faster rate at which it leaves the liver after various intervals of fasting. The rate of drop in liver fat is slower after the high cholesterol diet than in the case of the other fats.

Significant sex differences in the level of liver glycogen in unfasted rats were consistently noted in the tests on the various fat diets as well as in the control tests with the low fat diet.

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A PRECISE METHOD FOR THE DETERMINATION OF COUMARIN, MELILOTIC ACID, AND COUMARIC ACID IN PLANT TISSUE*

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The relatively unpalatable nature of the *common sweet clovers*, *Melilotus alba* and *Melilotus officinalis*, and their tendency to become toxic to live stock if improperly cured has stimulated biochemical studies in this genus. Recently a *non-bitter sweet clover* was found by Brink (1) which proved to be an annual form of *Melilotus dentata*. This led to cooperative studies¹ which have as their ultimate objective the improvement of the agricultural usefulness of sweet clover by developing more palatable strains which are also free of the tendency to become toxic.

A thorough qualitative study of the two species of *common sweet clover* shows a marked difference in chemical composition as compared with the *non-bitter Melilotus dentata*. We have found that the seeds and green tissue of the *bitter* species contain large quantities of coumarin, usually smaller amounts of melilotic acid, and only traces of coumaric acid. The seeds of *Melilotus dentata*, on the other hand, contain only small amounts of coumarin. Melilotic acid and coumaric acid, if present, exist only in traces. The green tissue of *Melilotus dentata* appears to contain, at the most, only minute traces of the three compounds.

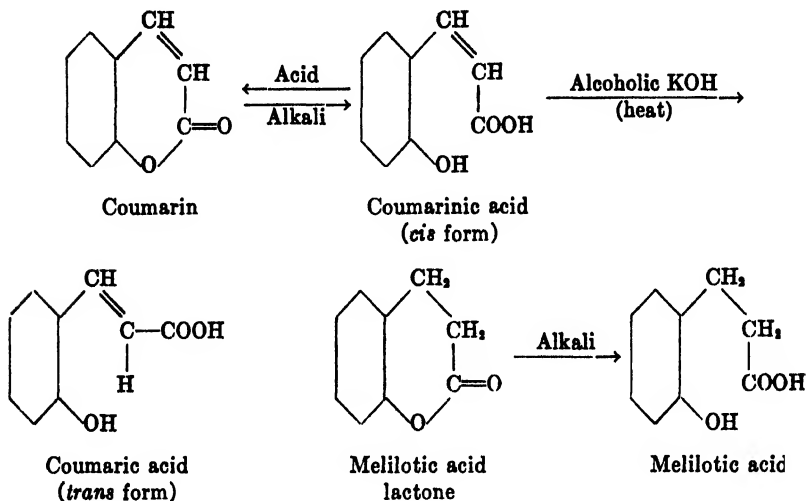
In order to determine the relationship of these substances to the palatability of the fresh tissue and the toxicity of the spoiled hay, a quantitative procedure for measuring them had to be developed.

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

¹ Cooperative studies with the Department of Genetics, University of Wisconsin, and the Division of Forage Crops and Diseases, United States Department of Agriculture.

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It is clear from the accompanying formulæ that the determination of three compounds so closely related in chemical structure presents a formidable problem.



Methods for the determination of coumarin or coumarin and melilotic acid have been described by others. However, it was soon learned in the course of this study that none of the existing procedures was capable of the degree of precision and accuracy demanded in this work.

The permanganate oxidation method originally developed by Obermayer (2) for the determination of coumarin has been shown to be faulty. Stevenson and Clayton (3) showed there is considerable loss of coumarin in the preparation of a sample dry enough for ether extraction, and Duncan and Dustman (4) proved that destruction of coumarin takes place in the prolonged ether extraction.

The gravimetric procedure developed by Kanewskaja and Fedorowa (5) for the determination of coumarin and melilotic acid is not only subject to the inherent weaknesses of Obermayer's method, but is also rendered unreliable by the fact that the melilotic acid fraction is grossly contaminated with other organic acids and plant pigments.

Duncan and Dustman (4) improved Obermayer's method to some extent, but found it desirable to retain the tedious step of

removing the coumarin from the tissue by steam distillation, which, in their procedure is repeated six times.

The method developed by Clayton (6), which was subsequently slightly revised by Clayton and Larmour (7) and Stevenson and Clayton (3), is based on the formation of a red dye when diazotized *p*-nitraniline is coupled with coumarin and related compounds in an alkaline solution. This method is rapid and valuable for certain types of work but has two pertinent handicaps. It does not present a procedure which enables the complete separate estimation of coumarin and related compounds. Secondly, these substances are measured under conditions that do not eliminate the errors introduced by the presence of interfering plant pigments and certain phenolic bodies.

The colorimetric method described below is a modification of a gravimetric procedure developed by us² (unpublished) which gave satisfactory results but was too slow and involved too large a sample. In the colorimetric procedure the finely ground green tissue or seed is first extracted with acidulated 10 per cent aqueous acetone. The extracted coumarin, melilotic acid, and coumaric acid are separated from the original acetone solution by continuous extractions with selective solvents. The amount of the individual components (coumarin, melilotic acid, and coumaric acid) are finally measured colorimetrically after coupling with the diazonium solution introduced for the purpose by Clayton *et al.* (6).

EXPERIMENTAL

Description of Apparatus Employed—Pyrex test-tubes. 25 × 200 mm. marked for 50 ml. and 30 × 200 mm. unmarked.

Shaking machine. The motion of the shaking machine should be such as to provide a back and forth movement of the extraction solution-green tissue mixture in the test-tubes.

Liquid-liquid extractors (8). These are conveniently prepared from 25 mm. Pyrex tubing. The length over all is 400 mm.; the 12 mm. side arm is exactly centered on the length of the tube. The inner tube, length 410 mm., is prepared from 7 mm. Pyrex tubing. One end carries a bulb perforated with a few small equally spaced holes; the other end a funnel of appropriate size.

² The gravimetric procedure developed can be used for the isolation and characterization of coumarin and related substances and will be described in a separate paper.

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The holes in the bulb must be small, equal in size, and in the same plane, so that many small bubbles of ether will be evolved rather than single large bubbles from the largest hole. Each extractor is fitted with a West condenser approximately 750 mm. in length. The 125 ml. Erlenmeyer flask containing the solvent is heated in a beaker of water which is in turn heated by a small flame.

Solvents and Solutions—

Petroleum ether. B.p. not over 40°.

Ethyl ether. Washed with three consecutive portions of water for the removal of most of the alcohol.

Extraction solution. 1 volume of acetone and 9 volumes of approximately 0.1 N H_2SO_4 .

Standard coumarin, melilotic acid, and coumaric acid solutions. Each contains 0.1 mg. per 1 ml. of solution.

p-Nitraniline hydrochloride (Solution A). Dissolve 3.5 gm. of *p*-nitraniline in 45 ml. of 37 per cent hydrochloric acid, dilute to 500 ml. with distilled water, and filter. This solution keeps indefinitely if stoppered.

Sodium nitrite (Solution B). Dissolve 5 gm. of sodium nitrite in 100 ml. of distilled water. Keep this solution in a dark bottle away from light and renew it frequently.

Diazonium solution. Thoroughly chill a 100 ml. flask and Solutions A and B in chipped ice. Now pipette 3 ml. of Solution A and 3 ml. of Solution B into the 100 ml. flask, chill for 5 minutes, add 12 ml. of Solution B, swirl, chill for another 5 minutes, fill to the mark with ice-cold distilled water, mix, and place in chipped ice for 15 minutes before using. If kept on ice, this solution will remain stable for 24 hours.

*Preparation of Color Standards—*Color standards, for comparison purposes, are prepared by pipetting the requisite amounts of the standard solutions in question into test-tubes marked for 50 ml. 5 ml. of 1 per cent Na_2CO_3 solution and sufficient distilled water to make a volume of approximately 40 ml. are then added. The coumarin standards are then heated for 15 minutes at 85°, after which they are cooled. The melilotic acid and coumaric acid standards are not heated. Now add 5 ml. of the diazonium solution, make to volume, mix, let stand for 2 hours, and use for comparison purposes. The standards are good for several days after the color is developed.

Analytical Procedure

Preparation of Sample—The sample should consist of at least 5 gm. of fresh green tissue. This material is sliced as finely as possible and is thoroughly mixed prior to weighing the portions for analysis. When seeds are to be analyzed, they should be ground in a mortar, and the ground mass well mixed.

Determination of Moisture—Weigh approximately 1 gm. to ± 0.001 gm. into a tared, flat bottom dish. Place in an air oven maintained at 100-105° for 4 hours. Cool and weigh. From the loss in weight calculate the per cent moisture.

*Preparation of "Solution for Analysis"*³—Weigh a 3 gm. portion of sliced tissue or 1.5 gm. of ground seeds to ± 0.001 gm. and transfer to a 100 ml. test-tube containing exactly 50 ml. of the extraction solution; close with a rubber stopper and start the shaking.

When the results of the moisture determination are obtained, remove the tube from the shaking machine and, using a burette, add an amount of the extraction solution that will make a total of 25 ml. when added to the volume of water contained in the sample involved. Now add a pinch of dry asbestos, close with the rubber stopper, and shake for a *total* of 24 hours.

After shaking, filter with suction through a small Buchner funnel fitted with a disk of qualitative filter paper and a mat of asbestos.⁴ Transfer 50 ml. of the clear filtrate (representing 2 gm. of plant tissue or 1 gm. of seeds) to the extractor and extract for 2 hours with ethyl ether.⁵ Remove the inner tube and transfer the ether layer in the extractor to the extraction flask.⁶ Now add 20 ml. of distilled water to the ether extract and carefully evaporate the ether by swirling the flask in a water bath (50-55°). Heat the aqueous solution to about 70° to dissolve water-soluble substances

³ See "Addendum."

⁴ The Buchner funnel must be dry. During the filtration it is often necessary to scratch the surface of the asbestos to increase the rate of filtration. The extract of ground seeds cannot be filtered. In this case centrifuge and take a 50 ml. aliquot of the turbid supernatant liquid.

⁵ Since superheating of the ether may cause decomposition, the 125 ml. flask should be immersed in the water bath (kept at 80°) in such a way that one-half of the ether in the flask is above the surface of the water.

⁶ This is easily done by displacing the ether by means of a carefully inserted test-tube of the proper size.

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and cool. Filter through a small qualitative filter paper into a 125 ml. flask and wash the original flask and the filter with 20 ml. of distilled water. The filtrate constitutes the "solution for analysis."

Separate Determination of Coumarin, Melilotic Acid, and Coumaric Acid

Coumarin—Add 0.5 ml. of 5 N NaOH to the solution for analysis, heat just to boiling, cool, add 0.75 ml. of 5 N H_2SO_4 , mix thoroughly, add 0.25 gm. of anhydrous NaHCO_3 , mix, and transfer to the extractor. Rinse the flask with distilled water and transfer to the extractor until the volume is 50 ml. Extract immediately for 3 hours with petroleum ether, remove the inner tube, and transfer the petroleum ether in the extractor to the extraction flask. Add 20 ml. of water to the petroleum ether extract and carefully evaporate the ether by swirling the flask in a water bath (50–55°). Transfer the aqueous solution to a 50 ml. volumetric flask, make to volume, and mix. Pipette a 25 ml. aliquot into a test-tube graduated for 50 ml., add 5 ml. of 1 per cent Na_2CO_3 solution, heat in a water bath at 85° for 15 minutes, and cool. Add 5 ml. of the diazonium solution, make to volume, mix, let stand 2 hours, and compare in a colorimeter against a coumarin standard of approximately the same color intensity. If the coumarin content of the 25 ml. aliquot exceeds 0.8 mg., repeat the color development with a smaller aliquot. Calculate the percentage of coumarin to the dry basis.

Melilotic Acid—Now add 1 ml. of 5 N H_2SO_4 to the aqueous solution remaining in the extractor, mix, extract for 2 hours with ethyl ether, remove the inner tube, and transfer the ether in the extractor to the extraction flask. Carefully filter the ether extract through a small dry filter paper into a dry 125 ml. flask. Wash the original flask and filter thoroughly with ether and evaporate just to dryness.⁷ Add 5 ml. of benzene and with occasional swirling boil for a few minutes on a steam cone. Cool for 5 minutes in chipped ice and let stand at room temperature for about 30 minutes. Now carefully decant the benzene extract through a small retentive filter paper (Whatman No. 44 or its equivalent) into a 50 ml. separatory funnel and wash the inside of the flask

⁷ Water must be absent at this point.

and the filter three times with a small jet of benzene. The total filtrate, washings included, should not exceed 15 ml. of benzene. This extract contains the melilotic acid. The Erlenmeyer flask and filter paper contain the coumaric acid. Add 15 ml. of 0.2 per cent Na_2CO_3 solution to the benzene extract and shake vigorously. Let stand a few minutes and draw off the aqueous layer³ through a qualitative filter paper into a 50 ml. volumetric flask. Repeat the shaking and drawing off process with a fresh 10 ml. portion of 0.2 per cent Na_2CO_3 solution and follow with 10 ml. of distilled water. Wash the paper and the funnel with distilled water, make to volume, and mix. This solution contains 5 ml. of 1 per cent Na_2CO_3 solution. Pipette a 25 ml. aliquot into a test-tube marked for 50 ml., add 2.5 ml. of 1 per cent Na_2CO_3 solution, and mix. Add 5 ml. of the diazonium solution, make to volume, mix, let stand 2 hours, and compare in a colorimeter against a melilotic acid standard of approximately the same color intensity. If the melilotic acid content of the 25 ml. aliquot exceeds 0.8 mg., repeat the color development with a smaller aliquot and the proper amount of 1 per cent Na_2CO_3 solution. (For suitable color development 5 ml. of 1 per cent Na_2CO_3 solution is necessary for the 5 ml. of diazonium solution.) Calculate the percentage of melilotic acid to the dry basis.

Coumaric Acid—Free the coumaric acid-containing Erlenmeyer flask and filter from adhering benzene by drying in a steam oven. Add 5 ml. of 1 per cent Na_2CO_3 solution to the flask, swirl, and pass through the filter into a test-tube marked for 50 ml. Wash the flask and filter with distilled water until the volume in the test-tube is about 40 ml., add 5 ml. of the diazonium solution, make to volume, mix, let stand 2 hours, and compare against coumaric acid standards of approximately the same color intensity. Calculate the percentage of coumaric acid to the dry basis.

Coumarin Singly, Melilotic Acid, and Coumaric Acid in Terms of Melilotic Acid

Using the "solution for analysis" determine coumarin as directed in the procedure above. After the extraction of coumarin add 1 ml. of 5 N H_2SO_4 to the aqueous solution remaining in the

³ Occasional emulsions may be broken by agitation of the affected area with a glass rod.

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extractor, mix, extract for 2 hours with ethyl ether, remove the inner tube, and transfer the ether in the extractor to the extraction flask. Add 20 ml. of water to the ether extract and carefully evaporate the ether in the usual manner. Transfer to a 50 ml. volumetric flask, make to volume, and mix. Couple an aliquot with the diazonium solution as directed under the procedure above for melilotic acid and compare against melilotic acid standards. Calculate to the dry basis and report as melilotic acid and coumaric acid in terms of melilotic acid.

Coumarin, Melilotic Acid, and Coumaric Acid in Terms of Coumarin

Add 0.5 ml. of 5 N NaOH to the solution for analysis, heat just to boiling, cool, and add 1 ml. of 5 N H₂SO₄; mix thoroughly and transfer to the extractor. Rinse the flask with distilled water and transfer to the extractor until the volume is 50 ml. Extract for 2 hours with ethyl ether, remove the inner tube, and transfer the ethyl ether in the extractor to the extraction flask. Add 20 ml. of distilled water to the ether extract and carefully evaporate the ether in the usual manner. Transfer to a 50 ml. volumetric flask, make to volume, and mix. Couple an aliquot with the diazonium solution as directed under the procedure for coumarin and compare against coumarin standards. Calculate to the dry basis and report as coumarin, melilotic acid, and coumaric acid in terms of coumarin.

Results

Table I shows that when the method is applied to pure solutions of coumarin, melilotic acid, and coumaric acid in admixture with each other accurate recoveries for the first two substances are realized over the entire range. With coumaric acid the accuracy drops off when the concentration falls below 0.1 mg.

Table II shows good recoveries when the pure compounds are added to a plant extract. Alfalfa tissue was used in this case, because it gave less red color in the various fractions than the particular *Melilotus dentata* on hand at the time the analyses were made. Here again accuracy and precision are realized except when the coumaric acid content is below a certain level.

Some typical results are shown in Table III. It is interesting

to note that *Melilotus dentata* seeds contain a small amount of coumarin, whereas the foliage appears to be devoid of this compound.

Table IV shows similar results obtained by our colleague Mr. Mark Stahmann in additional control determinations. One

TABLE I

Recovery of Varying Quantities of Coumarin, Melilotic Acid, and Coumaric Acid When in Admixture with Each Other in Acidulated 10 Per Cent Acetone Solution

Run No.	Coumarin		Melilotic acid		Coumaric acid	
	Added	Found	Added	Found	Added	Found
	mg.	mg.	mg.	mg.	mg.	mg.
1	25.12	25.10	2.51	2.51	2.02	1.99
2	10.05	9.98	1.00	1.04	1.00	0.98
3	2.00	2.00	1.00	1.05	1.00	0.98
4	0.10	0.099	1.00	1.04	0.50	0.48
5	0.10	0.099	0.10	0.140	0.10	0.036

TABLE II

Recovery of Varying Quantities of Coumarin, Melilotic Acid, and Coumaric Acid When Added to Acidulated 10 Per Cent Acetone Extract of 2 Gm. of Alfalfa Tissue

Run No.	Coumarin			Melilotic acid			Coumaric acid		
	Added	Found	Corrected*	Added	Found	Corrected*	Added	Found	Corrected*
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	20.08	20.10	20.07	2.64	2.83	2.65	1.23	1.33	1.25
2	5.02	5.00	4.97	0.88	1.14	0.96	1.24	1.33	1.25
3	0.10	0.124	0.097	5.54	5.71	5.53	0.61	0.64	0.56
4	0.10	0.134	0.107	0.100	0.280	0.100	0.100	0.109	0.029
5	None	0.027		None	0.180		None	0.080	

* Corrected for the blank obtained with alfalfa control tissue to which no coumarin, melilotic acid, or coumaric acid had been added.

sample of *Melilotus officinalis* analyzed in quadruplicate shows that the method gives reproducible results. The remaining data in Table IV also show the accuracy of the method. This sample of *Melilotus dentata* contained many seed pods which are probably responsible for the small percentage of coumarin. The *Melilotus dentata* in Table III carried no seed and contained no coumarin.

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TABLE III

Coumarin, Melilotic Acid, and Coumaric Acid Content of Green Tissue and Seeds of Various Species of Melilotus

Sample	Moisture	Coumarin	Melilotic acid	Coumaric acid
Green tissue				
	per cent	per cent on dry basis	per cent on dry basis	per cent on dry basis
<i>M. alba</i> (Hubam), 1st cutting July 30.....	72.1	2.08	0.14	0.010
<i>M. dentata</i> (annual).....	72.3	0.0000	0.027	0.033
Alfalfa (control).....	70.9	0.0047	0.030	0.013
Seed				
	per cent	per cent on dry basis	per cent on dry basis	per cent on dry basis
<i>M. officinalis</i> (biennial).....	6.76	0.63	0.023	0.0059
" <i>alba</i> (biennial)	6.81	0.46	0.020	0.0030
" <i>dentata</i> , Ac-85 (biennial)	6.87	0.074	0.012	0.0088
" " Ac-89 "	6.70	0.040	0.012	0.0088
" " (annual).....	8.32	0.021	0.0087	0.0054
Alfalfa (control).....	6.95	0.0058	0.0075	0.0053

TABLE IV

Analyses by Independent Worker, Unfamiliar with Analytical Procedure, Showing Reproducibility and Accuracy of Method

Sample	Moisture	Coumarin on dry basis	Melilotic acid on dry basis	Coumaric acid on dry basis
	per cent	per cent	per cent	per cent
<i>M. officinalis</i>	70.8	0.69	0.043	0.014
" "	70.8	0.69	0.045	0.014
" "	70.8	0.68	0.041	0.020
" "	70.8	0.69	0.043	0.016
" " A-9.....	69.5	0.64	0.24	0.035
" " A-9	69.5	0.67	0.26	0.038
" <i>alba</i> (Hubam), 2nd cutting Oct. 20.	77.7	0.086	0.40	0.035
" " " 2nd " " 20.	77.7	0.088	0.40	0.031
" <i>alba</i> , A-10.....	69.1	0.36	0.27	0.048
" " A-10.....	69.1	0.37	0.27	0.062
" " A-43.....	73.5	0.93		0.018
" " A-43.....	73.5	0.97	0.28	0.019
" <i>dentata</i> (annual).....	75.0	0.018	0.026	0.042
" " "	75.0		0.025	0.035
Alfalfa (control).....	71.2	0.0041	0.025	0.025
" "	71.2	0.0080	0.026	0.025

DISCUSSION

The determination of coumarin, melilotic acid, and coumaric acid as outlined in this paper gives satisfactory results with *common sweet clover*. The red colors in the coumarin and melilotic acid fractions are easily compared against the proper standards. The coumaric acid fraction may offer some difficulty owing to the presence of a yellow color. This difficulty can be largely avoided if the reader concentrates on the intensity of the red color while making the color comparisons.

The results obtained in the analysis of alfalfa and *Melilotus dentata* appear to be somewhat in error. In the case of *Melilotus dentata* seeds coumarin is responsible for the red color obtained in the coumarin fraction. We believe that most of the red color produced in the melilotic acid and coumaric acid fractions is due to the presence of other phenolic bodies. The red colors developed are of a different shade than those produced by the pure compounds. Some of the naturally occurring compounds which we have been able to couple to form a red dye are *p*-hydroxybenzoic acid, gallic acid, guaiacol, phenol, phloroglucinol, protocathechuic acid, pyrocatechol, pyrogallol, thymol, and vanillic acid. It is possible that one or more of these compounds or others are responsible for the traces of red color.

In comparative studies on different species of *Melilotus*, the traces of coumarin and the small amounts of phenolic bodies contaminating the melilotic acid and coumaric acid fractions can be disregarded. The real interest centers in the use of the method with common sweet clovers which contain appreciable amounts of coumarin, melilotic acid, and coumaric acid which can be measured accurately by the method described.

The plant geneticist and forage crop specialist thereby have at their disposal a chemical method for the absolute and comparative estimation of coumarin and related substances in studies dealing with the selection of species or strains of *Melilotus*.

SUMMARY

A colorimetric method for the determination of coumarin, melilotic acid, and coumaric acid in small amounts of sweet clover tissue and accurate for the range in which these constituents occur has been described.

The method is based on the principle of first extracting the components from the green tissue or seeds with acidulated aqueous acetone (10 per cent). After each component has been separated from the crude extraction mixture, through the use of a selective solvent, it is coupled with a diazonium solution prepared from *p*-nitraniline. The coupling procedure produces a red dye which lends itself to a colorimetric estimation in an ordinary colorimeter. The color comparisons are made against known standards of coumarin, melilotic acid, and coumaric acid produced by coupling the pure substances with the diazonium reagent.

Summarized tabulations showing the performance of the method with varying amounts of pure coumarin, melilotic acid, and coumaric acid in admixture with each other and when added to alfalfa tissue as control are given. The analyses of various species of sweet clover and alfalfa tissue for coumarin, melilotic acid, and coumaric acid are presented. It is shown that the method can be employed to differentiate between so called *bitter* and *non-bitter* species of *Melilotus* on the basis of their coumarin, melilotic acid, and coumaric acid content.

The writers wish to express their thanks and appreciation to Dr. E. A. Hollowell of the United States Department of Agriculture, Washington, and Professor R. A. Brink and Dr. W. K. Smith of the Genetics Department of this institution for financial assistance and counsel. The help of Mr. Mark Stahmann, Graduate Training Fellow, on control determinations is also gratefully acknowledged.

Addendum—After the above communication was submitted, it was learned that the estimation of coumarin content of sweet clover tissue and sweet clover seeds (particularly the latter) can be placed on a much firmer basis by including an incubation period for the enzymatic release of bound coumarin. Proceed as follows: incubate the 1.50 gm. of ground tissue or 3.0 gm. of the finely minced green tissue with 10 ml. of water (in the closed test-tube) at 40° for 1 hour. After the incubation period the mixture is made to the desired volume with the extraction solution. Thorough shaking and filtering in the manner described yield the "solution for analysis."

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THE EFFECT OF CERTAIN PHYSIOLOGICALLY IMPORTANT MATERIALS UPON KIDNEY PHOSPHATASE

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(Received for publication, January 23, 1937)

This investigation was undertaken to complete a general survey of the effect of certain vitamins, hormones, and blood constituents upon the activity of phosphatase. Some of these vitamins and hormones control physiological processes and developments which appear to be related to phosphatase activity; others, which had no apparent physiological connection, were also investigated to cover the field adequately.

Heymann (1) reports that parathyroid hormone in a concentration of 0.1 unit per cc. of hydrolyzing mixture inhibits bone phosphatase activity, *in vitro*, from 50 to 100 per cent. The effect was less in the case of kidney and intestinal phosphatase. Bakwin and Bodansky (2) repeated these experiments upon bone extracts, using up to 2.5 units per cc. of hydrolyzing mixture, and found no effect.

Heymann (1) investigated the effect of irradiated ergosterol upon phosphatase *in vitro* and reported no effect for 1 or 2 drops of vigantol per cc. of hydrolyzing mixture. No information was given as to the concentration of the drops. In this investigation, by using a 60 per cent acetone hydrolyzing mixture, concentrations of calciferol ranging from 0.00001 to 0.12 mg. per cc. were obtained without difficulty.

EXPERIMENTAL

The source of the enzyme was a dry preparation obtained from swine kidneys, according to the procedure of Albers and Albers (3). This was shaken overnight with distilled water, filtered, and kept in an ice box.

TABLE I

Material	pH	Experi- ment No.	No. of sam- ples	Concentration per cc. hydrolysing mixture*	Activity	Conclusions
Parathyroid	7.3	1-a	7	2 -0.001†	Same as control	No effect
"	9.0	1-b	9	2.5 -0.001†	" "	"
Calciferol	7.3	2	9	0.12-0.00001	" "	"
Insulin	9.0	3-a	18	2 0 -1 × 10 ⁻⁴ †	" "	"
"	7.3	3-b	6	1 0 -0.003†	" "	"
Adrenalin	7.3	4-a	7	0.5 -0.002	" "	"
"	7.3	4-b	10	0 8 -0.00001	" "	"
Pituitary						
Anterior lobe	7.3	5-a	7	1 0 -0.00032†	" "	"
Posterior "	7.3	5-b	8	0 1 -0.0000013†	" "	"
Sex factor	7.3	5-c	10	5 0 -0.001†	" "	"
Acetylcholine bro- mide	7.3	6	10	0.5 -1 × 10 ⁻⁷	" "	"
Ascorbic acid	7.3	7	7	10‡	0 073 mg. P; inhibition 34%	Inhibition
			4	1 5	0.092 " " "	at higher
			0 5	0.095 " " "	0.095 " " "	concen-
			0.1	0.106 " " "	0 106 " " "	trations
			0.02	0.112 " " "	0.112 " " "	
			0 005	0.108 " " "	0 108 " " "	
			0	0.111 " " "	0.111 " " "	
Vitamin A	7.3	8	7	0 6 -4 × 10 ⁻⁵	Same as control	No effect
Bios I (inosite)	7.3	9-a	6	2 -0.00016	" "	"
" II-a	7.3	9-b	6	0 5 -0.00032§	" "	"
Butyric acid	7.3	10	7	10	0.52 mg. P; inhibition 63%	Inhibition
			1	0.1	1.16 " " "	
			0 01, 0.001, 0.00001, 0.00001	1.36 " " "	16%	
				1.40 " " "	3%	

Lactic acid Cystine	7.3 7.3	11 12	6 7	20, 2, 0.2, 0.02, 0.002, 0.0002 1.0 0.1 0.04 0.01 0.001, 0.0001, 0.00001 1.5-0.00015 20-0.0002 0.25-0.0025 20-0.5 10-0.0002 10-0.0002 5-0.0002 7 2 0.2 0.02 0.002 0.0002 0 20 2 0.2 0.02 0.002 0.0002 0	Same as control 0.109 mg. P; inhibition 39% 0.150 " " " 16% 0.135 " " " 26% 0.167 " " " 7% 0.179 " "	No effect " " " " " " " " " " " " Activation over whole range Maximum activation occurs in this range
Cystine Alanine Tyrosine Glycine Leucine Aspartic acid Tryptophane Creatine	7.3	13	6		Same as control " " "	No effect " " " " " " " " " "
	7.3	14	6		" " "	" "
	7.3	15	3		" " "	" "
	7.3	16	6		" " "	" "
	7.3	17	5		" " "	" "
	7.3	18	6		" " "	" "
	7.3	19	6		" " "	" "
	7.3	20	6		0.228 mg. P; activation 31% 0.217 " " " 24% 0.222 " " " 27% 0.214 " " " 22% 0.209 " " " 18% 0.204 " " " 15% 0.181 " " " 7% 0.192 " " " 16% 0.206 " " " 14% 0.203 " " " 14% 0.209 " " " 18.5% 0.195 " " " 9% 0.181 " " "	Activation over whole range Maximum activation occurs in this range
	7.3	21	6			
Creatinine	7.3					

* The values are given in mg. except where otherwise noted.

† Measured in units per cc.

‡ Corrections made for the effect of ascorbic acid upon the phosphorus determination.

§ Measured in cc. per cc.

A solution was made up containing substrate (3.66 per cent sodium β -glycerophosphate) and buffer (1.5 per cent sodium diethylbarbiturate) and adjusted colorimetrically to pH 7.3 or 9.0. 3 cc. of this solution were used in the 10 cc. hydrolyzing mixture. All solutions to be added to the hydrolyzing mixture were brought to the same pH to guard against any shift.

The enzyme activity was determined by measuring the inorganic phosphate produced, with the colorimetric method of Kuttner and Cohen (4) and the correction tables of Bodansky (5). The activity is expressed in mg. of phosphorus hydrolyzed in the 10 cc. of hydrolyzing mixture by 0.3 mg. of phosphatase. The organic phosphorus in 10 cc. was 18 mg.; inorganic phosphorus was too low to be determined accurately.

The reaction was carried out at a temperature of $37.0^{\circ} \pm 0.1^{\circ}$ and stopped by adding an equal volume of 7 per cent trichloroacetic acid.

To study the effect of the fat-soluble vitamins A and D, the hydrolysis was carried out in 60 per cent (volume) acetone, at which concentration the vitamins stayed in solution and the enzyme was only inhibited 50 per cent.

The calciferol was supplied by The British Drug Houses, Ltd., and contained 25,000 units per mg.

In Experiment 4-a (Table I) the adrenalin was oxidized even in 1.5 hours; so in Experiment 4-b, 1 cc. of 0.2 per cent sodium sulfite was added to 10 cc. of hydrolyzing mixture, which prevented oxidation. In Experiment 5-a the unit is equivalent to the extract from 12 mg. of anterior lobe. Bios II-a was prepared from alfalfa in the Department of Dairying, the University of British Columbia. In the experiments in which no effect is reported, all of the phosphate determinations agreed within 10 per cent, and the majority were within 5 per cent of the control value.

Bremner and Clark (6), studying the effect of the bioses on amylase activity, have shown that a mixture of Bios I, II-a, and II-b produces an activation 23 per cent greater than the sum of the activations when each is taken separately and at the same concentration. Mixtures of vitamins and hormones were used upon phosphatase but with no effect. Mixtures of (1) insulin-adrenalin, (2) parathormone-insulin, (3) parathormone-

adrenalin-anterior pituitary-adrenalin were tried, each at three different concentrations, but without effect. Mixtures of (1) vitamins A, C, D, parathormone, and antuitrin, (2) vitamin C, parathormone, adrenalin, and insulin, (3) thyroxine, antuitrin, pituitrin, and acetylcholine were tried, each at two concentrations which were near the calculated physiological concentrations, but again effects were negligible.

DISCUSSION

The results obtained with parathormone are in agreement with those of Bakwin and Bodansky (2) who found no effect upon bone phosphatase but contradict Heymann's (1) report of inhibition of kidney phosphatase.

In the case of vitamin D, Heymann (1) found irradiated ergosterol had no effect upon phosphatase *in vitro*, and these experiments, conducted over a much wider range and under better controlled conditions, agree with his observations.

Creatine and creatinine were found to produce a slight activation, whereas butyric acid, cystine, and vitamin C produced inhibition. In most cases, however, the concentrations necessary to produce these results are considerably above the physiological concentrations, even should there be adsorption of the material at the organ or the seat of the action.

Thus kidney phosphatase proves to be extremely stable and is little affected by other materials present in the body. This stability is rather remarkable, considering the variety of substances tried upon it and the large range of concentrations. This suggests that there may be only one or two definite accelerators such as magnesium ions, and not any one of a group as found in the case of amylase activity, which is accelerated by most halogen compounds (7).

SUMMARY

1. Of the vitamins and hormones tried, vitamin C produced inhibition, but parathormone, vitamins A and D, insulin, adrenalin, pituitary extracts of anterior and posterior lobes, and acetylcholine bromide had no effect.

2. Of the other materials creatine and creatinine produced activation; butyric acid and cystine produced inhibition; and Bios

I and II-a, lactic acid, cysteine, alanine, tyrosine, glycine, leucine, aspartic acid, and tryptophane had no effect.

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COMPARISON OF THE DISTRIBUTION OF MAGNESIUM IN BLOOD CELLS AND PLASMA OF ANIMALS

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(Received for publication, March 29, 1937)

Greenberg and his associates (1) found that the magnesium content of human blood was considerably higher than that of plasma from the same source. These authors state that dog blood is very similar to human blood. In comparing their data with the recalculated magnesium data of Abderhalden (2), Greenberg *et al.* remark on the low cell magnesium of sheep and cattle blood cells found by Abderhalden. Few other data are available for comparing the cell and plasma magnesium of different species of animals. Greenberg and Mackey (3) found swine blood cell magnesium higher than the plasma magnesium. Green and Macaskill (4) reported analyses on the blood of a cow and a calf in which the cell magnesium was higher than the plasma.

As a part of routine analyses of whole blood and plasma of different species of animals under observation in this laboratory a large number of magnesium determinations have been made. A few typical analyses of the whole blood and plasma of normal individuals of the different species are reported.

Methods

3 mg. of sodium citrate per ml. of blood were used as an anti-coagulant. Trichloroacetic acid filtrates were made of both whole blood and plasma and the calcium precipitated as the oxalate at approximately pH 5.6. Aliquots of the Ca-free filtrates were transferred to 15 ml. centrifuge tubes and the magnesium precipitated as magnesium ammonium phosphate. After 24 hours the precipitate was centrifuged out and washed twice with 2 ml. portions of 1:2 dilution of ammonium hydroxide. After draining and drying, the phosphorus was determined colorimetrically (5).

290 Magnesium in Blood Cells and Plasma

TABLE I

Magnesium Content of Blood Cells and Plasma of Different Animals

The values are given in mg. per cent.

Species	Animal No.	Hematocrit	Whole blood	Plasma	Cells
		<i>per cent</i>			
Mouse*		32.8	10.3	7.6	14.5
Rat	1	42.0	5.9	2.6	10.5
	2	36.2	5.6	3.7	8.8
	3	42.8	5.1	3.7	7.0
	4	45.5	5.3	2.7	8.3
Guinea pig*		32.8	6.4	4.8	9.8
Rabbit	1	35.6	5.6	2.7	10.9
	2	44.7	4.4	2.8	6.5
	3	44.7	5.2	3.2	7.6
	4	45.0	5.1	2.6	8.2
	5	36.0	5.6	3.5	9.4
	6	27.9	4.9	3.1	9.7
	7	28.6	6.9	4.2	13.6
Chicken	1	35.6	4.9	3.2	7.9
	2	34.0	5.3	2.5	10.9
	3	30.7	5.3	2.2	12.0
	4	38.9	4.7	3.0	7.5
	5	35.3	4.9	3.2	7.9
Horse	1	37.5	3.2	2.2	4.8
	2	33.1	3.4	2.1	6.0
	3	32.0	3.5	2.0	6.6
	4	35.0	4.4	2.5	8.0
	5	28.7	3.6	2.5	6.3
	6	38.9	5.0	2.6	8.7
	7	32.4	3.4	2.2	5.9
	8	39.9	5.1	2.6	8.8
	9	34.3	3.8	3.0	5.3
	10	28.9	4.1	2.6	8.0
Swine	1	42.5	6.5	3.3	11.0
	2	42.5	6.6	3.4	10.9
	3	42.2	7.0	3.4	12.0
	4	41.8	7.5	3.3	12.0
	5	43.2	4.9	2.4	8.3
	6	41.6	5.0	2.4	8.5
	7	47.0	7.3	2.5	12.8
	8	45.6	5.7	3.8	10.0
	9	54.5	7.2	3.9	9.9
	10	45.0	6.2	3.4	9.5

* Pooled sample.

TABLE I—*Concluded*

Species	Animal No.	Hematocrit	Whole blood	Plasma	Cells
		<i>per cent</i>			
Goat	1	46.8	3.1	2.7	3.6
	2	39.5	4.2	3.6	4.3
	3	23.1	3.8	3.3	5.6
Sheep	1	34.4	3.4	2.8	3.9
	2	37.3	3.3	3.0	4.2
	3	41.4	3.3	2.9	3.3
	4	42.7	3.5	3.0	4.0
	5	38.8	3.1	2.9	3.3
	6	40.3	3.2	2.7	4.0
	7	42.7	3.1	3.0	3.3
	8	44.5	3.5	3.1	4.0
	9	33.0	3.3	2.9	4.2
	10	40.3	3.3	3.1	3.7
Dairy cattle	1	52.0	2.3	2.3	2.3
	2	46.5	2.3	2.5	2.1
	3	39.2	2.3	2.5	2.0
	4	43.5	2.3	2.4	2.0
	5	49.5	2.2	2.4	2.0
	6	42.2	2.0	3.0	0.7
	7	35.2	2.8	3.5	1.7
	8	41.8	2.5	3.0	1.9
	9	38.0	2.2	3.3	0.5
	10	44.5	3.0	3.2	2.9

In certain cases the magnesium ammonium phosphate method was compared with the hydroxyquinoline method of Greenberg and Mackey (3). The two methods agreed very well.

Hematocrit values were obtained by centrifuging to constant volume in Wintrobe (6) tubes. No correction was made for shrinkage in cell volume due to the anticoagulant.

DISCUSSION

The values recorded in Table I agree for the most part with the data of Abderhalden (2). A small number of analyses are recorded in Table I but in many cases numerous subsequent determinations have been made on the same individuals. Also analyses of the blood of numerous animals with various disease conditions have been compared with these data. The physiologic and

pathologic variations of blood magnesium are not understood. These data are presented to show the comparative distribution of magnesium in the cells and plasma of apparently normal individuals.

It is of interest to note that of all of the animals studied, cattle are the only ones that usually show higher plasma magnesium than cell magnesium. Occasionally a higher whole blood than plasma magnesium is encountered but it has been extremely rare. Sheep and goats show only slightly higher cell than plasma magnesium. When all of the data are compared with those of Abderhalden (2) and Greenberg *et al.* (1), it seems rather definitely established that low cell magnesium is confined to ruminants. Dietary factors could be partially responsible, but this seems unlikely. These data have been collected on animals from various sources. Analyses on twenty-eight range cattle show a whole blood average of 2.6 and a serum average of 2.8 mg. per cent, which indicates a slightly higher serum than cell magnesium in these cattle.¹

Individual cases in all species may show extremely wide deviations from average values. With the information available there does not appear to be any way of correlating the relation of cell to plasma magnesium.

SUMMARY

Data are presented on the distribution of magnesium in the cells and plasma of different species of animals.

The author wishes to thank Mr. T. W. Millen for assistance in some of these analyses.

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SOLUBILITY OF BONE SALT

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The purpose of this investigation was to determine at what concentrations of calcium and phosphate ions the salts of bone could be formed from solution. Because of the confusion which exists in the interpretation of data on the solubility of the calcium salts of bone, the facts pertinent to this problem and the argument of the present paper will first be outlined

Chemical Nature of Solid—When calcium and phosphate ions form a precipitate from neutral or alkaline solutions, a substance is formed which shows no definite crystalline structure with the microscope but by x-ray examination shows definite lines indicating that the material consists of a crystalline salt (1-5). The composition of the precipitate at first approximates $\text{Ca}_3(\text{PO}_4)_2$ (6, 7), but is variable, depending on the composition of the solution from which it is precipitated (7-9). When the precipitate is left in contact with the solvent, the composition of the precipitate is altered so that the ratio of calcium to phosphate is increased (7, 10). When bicarbonate is present in the solution, the precipitate will be found to contain CO_2 and extra calcium, the amount of which increases with the time of contact between the precipitate and the supernatant liquid, so that the precipitate may come to have a composition represented approximately by the formula $[\text{Ca}_3(\text{PO}_4)_2]_n\text{CaCO}_3$ (10).

The composition of the inorganic portion of bone corresponds with such a formula, except that approximately 6 per cent of the base consists of Mg, Na, and K (11). n is about 3 in the bone from young animals of some species, and approaches 2 in adult bone from most of the species which have been studied (11, 12).

The x-ray spectrograms of substances represented by the

formula $\text{Ca}_3(\text{PO}_4)_2$ or $[\text{Ca}_3(\text{PO}_4)_2]_2\text{CaCO}_3$, including the bone salt, are indistinguishable by the methods at present employed (2-5).

The precipitate obtained from the union of calcium and phosphate ions in slightly acid solution may be, at first, CaHPO_4 , which forms definite crystals and has an x-ray spectrogram distinctly different from $\text{Ca}_3(\text{PO}_4)_2$ or bone. In contact with water or solutions, the pH of which may be as low as 5.0, CaHPO_4 is slowly converted into tricalcium phosphate (6, 10).

The Argument—It is necessary to consider the chemical nature of the bone salt, because the nature of this salt depends on the steps involved in its precipitation. Two possibilities exist.

1. The bone salt may consist of large molecules composed of Ca , PO_4 , and CO_3 ions in stoichiometric proportion. Such an idea has persisted since the time of Hoppe (13-15). More recently (16) in studies of the solubility of the bone salt and of apatite minerals (which have a similar chemical composition), the same assumption is made. By inference, it is also assumed that the bone salt forms by simultaneous union and dissolves by simultaneous dispersion of the ions. The use of the solubility product $[\text{Ca}^{++}]^3 \times [\text{PO}_4]^{12} \times \text{CO}_3$ in these determinations implies that the precipitate forms by one step.

2. The other possibility, and the one which the data of this paper support, is that the precipitate forms by more than one step. For example, the precipitate which forms first may be $\text{Ca}_3(\text{PO}_4)_2$. Subsequently, it may adsorb additional ions such as Ca^{++} and CO_3^{--} from the liquid phase. (The presence of some of the ions in the precipitate might also be accounted for by solid solution.)

The evidence from the x-ray examination of the substances is consistent with either view-point. This is because substances adsorbed on the surface of a solid do not influence the x-ray pattern, and those in solid solution deflect the lines characteristic of the predominant substance so little that they are not detected by the methods which have been employed (17). Consequently, if the final precipitate forms by a process of adsorption on and solution in $\text{Ca}_3(\text{PO}_4)_2$, substances with the composition such as $[\text{Ca}_3(\text{PO}_4)_2]_2\text{CaCO}_3$ would be expected to give the same x-ray spectrogram as substances with the composition $\text{Ca}_3(\text{PO}_4)_2$.

The concentration of calcium and phosphate ions necessary to form the bone salt depends on the sequence of events by which the

precipitate forms. The evidence presented here, which is based on the solubility of the bone salt, is consistent with the hypothesis that $\text{Ca}_3(\text{PO}_4)_2$ forms first. Subsequent changes in the composition of the precipitate result, in part, from adsorption of ions from the liquid phase, and (possibly), in part, from solid solution of the ions.¹

The reasons are as follows: If the first hypothesis were correct—namely, if the bone salt consists of large molecules which form by simultaneous union of the ions—then the solubility product of the salt formed from supersaturated solutions should be essentially the same if a small or large amount of solid phase is brought into equilibrium with a liquid phase. Our data do not support this hypothesis.

On the other hand, if the precipitate first formed is $\text{Ca}_3(\text{PO}_4)_2$, and if it adsorbs, from the solution, ions of salts below their solubility products, it would follow that the ion product $[\text{Ca}^{++}]^3 \times [\text{PO}_4^{--}]^2$ required to form the precipitate would be greater than that at which the precipitate would dissolve. The equilibration of a supersaturated or undersaturated solution with a relatively large amount of solid would determine the ion product at which the solid would dissolve, but not that at which it would precipitate.

If ions are adsorbed by the first precipitate formed, it follows that the ion product $[\text{Ca}^{++}]^3 \times [\text{PO}_4^{--}]^2$ should increase as the amount of solid equilibrated with a given quantity of solution decreases. The solubility product of the $\text{Ca}_3(\text{PO}_4)_2$ could be determined only by equilibration with an amount of solid so small that it adsorbs a negligible amount of ions from the supernatant liquid. The results would represent the concentrations of ions at which precipitation could proceed in the absence of supersaturation. The precipitate could dissolve only when the ion concentrations were decreased below the concentrations in equilibrium with larger amounts of solid.

The "solubility products" of tricalcium phosphate to be found in the literature (6, 18) were obtained by equilibrating 1 gm. or more of solute per liter of solution. The results, when expressed

¹ Experiments were conducted primarily to study the relation of the composition of the precipitates to the composition of the solutions from which they were precipitated, and will be reported in detail separately (Table III).

as $p[(Ca^{++})^3 \times (PO_4^{=})^2]$ for $\mu = 0.155$, are usually 26.5 to 27.75 at 38°.² The ion products, expressed as $p[(Ca^{++})^3 \times (PO_4^{=})^2]$, obtained when 10 gm. or more of ground bone are equilibrated per liter of solution, vary between 25.5 and 26.0 (16). We have confirmed these results for tertiary phosphate when 150 mg. or more of the solid were found per liter of solution. When 100 mg. of bone per liter were equilibrated, the values for $p[(Ca^{++})^3 \times (PO_4^{=})^2]$ obtained were near those reported with large amounts. But we have found that the ion product at equilibrium progressively increases as the amount of solid used is decreased below those amounts, and, with minimum amounts, approaches the value 23.1 (± 0.4) for $p[(Ca^{++})^3 \times (PO_4^{=})^2]$.

EXPERIMENTAL

Methods

Calcium was determined by the method of Fiske and Logan (19), phosphate by the method of Fiske and Subbarow (20), carbon dioxide by the procedure of Van Slyke and Neill (manometric) (21), pH with a MacInnes glass electrode and a Leeds and Northrup potentiometer-electrometer, No. 7660. Preliminary adjustment of the pH during the experiments was aided by adding phenol red (10^{-4} per cent) and by comparison of the color of the solutions with that of phosphate buffers of similar ionic strength contained in flasks essentially similar to those used for equilibration. Calcium chloride was prepared from Kahlbaum's *zur Analyse* calcium carbonate. Sodium chloride was recrystallized from a solution saturated with hydrochloric acid. Merck's Blue Label dibasic sodium phosphate was used.

Calculation of Results— $PO_4^{=}$ ion concentrations were calculated from the equation $[PO_4^{=}] = (\text{total } (PO_4) K_1 K_2 K_3) / ((H^+)^2 K_1 + H^+ K_1 K_2)$. The values for K_1 , K_2 , K_3 employed were those calculated from the equations $pK_1 = 2.11 - 0.5 \sqrt{\mu}$, $pK_2 = 7.15 - 1.25 \sqrt{\mu}$, $pK_3 = 12.66 - 2.25 \sqrt{\mu}$ (18), where μ is the ionic strength of the solution. The Ca^{++} concentration was taken as equivalent to the total calcium concentration. The results are expressed on a molarity basis. Ions enclosed in

² The term $p[(Ca^{++})^3 \times (PO_4^{=})^2]$ represents the common logarithm of the reciprocal of the ion product $[Ca^{++}]^3 \times [PO_4^{=}]^2$.

brackets, [], refer to moles per liter of solution. pH values have been referred to the potential of the glass electrode containing standard acetate (0.1 M sodium acetate + 0.1 M acetic acid), assuming its pH to be 4.62 (22).

Results

Solubility Products with Small Amounts of Solid—In our early determinations of the solubility product, solutions were prepared from NaCl, Na_2HPO_4 , CaCl_2 , phenol red (10^{-4} per cent), and HCl so that the ionic strength was approximately 0.155 and the pH 7.0 to 8.0. The $[\text{Ca}^{++}]$ and $[\text{PO}_4^{=}]$ were sufficiently high to cause spontaneous precipitation. The precipitates were centrifuged, and the supernatant liquids were poured into other vessels and inoculated with a small portion of the previous precipitate. The pH of each solution was adjusted to the original value with NaOH and the process repeated until no additional precipitation was observed. The flasks were then again inoculated and rotated at constant temperature in a water bath at $25^\circ (\pm 0.1)$.

After 4 days, and again after 8 days, the pH of each solution was determined and a portion was filtered through tight ashless paper for calcium and phosphate analysis. The ion products found, expressed as $p[(\text{Ca}^{++})^3 \times (\text{PO}_4^{=})^2]$, were 23.43 (pH 7.15), 22.91 (pH 7.35), 23.37 (pH 8.10) in 4 days, and 23.52, 22.95, and 23.37,³ respectively, for the same solutions in 8 days. The agreement between the results obtained in 4 days and those in 8 days indicates that the precipitates had apparently essentially attained equilibrium.

Ion Products When Varying Amounts of Solid Were Formed—Experiments were designed to study the ion products at equilibrium, obtained when varying amounts of calcium phosphate precipitated from supersaturated solutions. These were carried out at $37.5^\circ (\pm 1^\circ)$ in a constant temperature room. Solutions containing NaCl, Na_2HPO_4 , HCl, CaCl_2 , phenol red (10^{-4} per cent), and in several cases NaHCO_3 (10 or 25 mM per liter) were inoculated with a few crystals of a calcium phosphate precipitate. The precipitate had been prepared by adding CaCl_2 (0.05 M)

³ Calculated with the dissociation constants adopted throughout the rest of the work.

to a mixture of Na_2HPO_4 (0.05 M) and NaHCO_3 (0.025 M) kept between pH 7.4 and 7.8 with NaOH during the addition. It was quickly washed once with water by centrifuging, but not dried. (The reason that further washing was avoided will be apparent later.)

The pH and the concentrations of calcium and phosphate in the solutions to be equilibrated were adjusted so that varying amounts

TABLE I

Determination of Solubility Product of $\text{Ca}_3(\text{PO}_4)_2$ by Formation of Varying Amounts of Solid from Supersaturated Solution

Temperature of equilibration 38° . All the solutions were prepared from CaCl_2 , Na_2HPO_4 , and HCl added to bring to the recorded pH and NaCl to make the ionic strength approximately 0.155.

Experiment No.	Solid formed	Duration of equilibration	Composition of solutions after equilibration			$p[(\text{Ca}^{++})^3 \times (\text{PO}_4^{=})^2]$
			pH	Ca	P	
	mg. per l.	days		mM per l.	mM per l.	
83*	<1	8	7.27	0.529	6.05	23.47
85†	<1	8	7.34	0.535	5.89	23.23
88†	<1	8	7.20	1.034	2.98	23.41
25‡	2 5	8	7.40	0.306	6.15	23.86
23‡	10	7	7.48	0.233	8.06	23.83
22‡	13	7	7.42	0.204	5.85	24.39
24	13	7	7.46	0.202	9.20	23.93
95†	47	5	7.82	0.055	5.89	25.26
19	57	10	8.10	0.039	5.64	25.10
26	95	7	7.27	0.082	5.35	26.02
98	99	5	8.10	0.043	2.51	25.67
27	167	7	7.38	0.015	7.20	27.71

* Other values of this point determined were 23.22, 23.36, 23.22.

† The solution contained 25 mM of NaHCO_3 per liter.

‡ The solution contained 10 mM of NaHCO_3 per liter.

of precipitate formed. When precipitation took place, the pH values of the solutions were readjusted to their original values with small amounts of NaOH and kept as nearly as possible constant throughout the equilibration. The Pyrex flasks containing the solutions were tightly stoppered and rotated continuously for 6 to 10 days. For the purpose of calculating the approximate amounts of precipitate formed, they were assumed to have the

formula $\text{Ca}_3(\text{PO}_4)_2$. The amounts were calculated as mg. of $\text{Ca}_3(\text{PO}_4)_2$ from the difference between the amount of calcium added and that found in the supernatant liquid after equilibration (Table I).

Ion Products When Varying Amounts of Solid Were Added—Finally, a solution of ionic strength 0.155, of which the ion product, $p[(\text{Ca}^{++})^3 \times (\text{PO}_4^{=})^2]$, was about 22, and of which the bicarbonate concentration was 25 mm per liter (Table II), was prepared and

TABLE II

Determination of Solubility Product of $\text{Ca}_3(\text{PO}_4)_2$ by Equilibration of Slightly Supersaturated Solutions with Varying Amounts of Added Solid

Composition of the solution at the start, CaCl_2 1.06 mm per liter, Na_2HPO_4 3.06 mm per liter, NaHCO_3 25.0 mm per liter, NaCl 120 mm per liter, HCl to pH 7.4. Temperature 38° ; duration of equilibration 8 days.

Experiment No.	Solid added Bone	Calculated as $\text{Ca}_3(\text{PO}_4)_2$	Composition of solutions after equilibration			$p[(\text{Ca}^{++})^3 \times (\text{PO}_4^{=})^2]$
			pH*	Ca	P	
	mg per l.	mg. per l.		mm per l.	mm per l.	
61	1.3	0.74	7.43	1.056	3.06	22.77
62	5.0	2.87	7.38	0.929	2.98	23.09
63	25.0	14.3	7.34	0.619	2.79	23.74
64	49.0	28.1	7.29	0.467	2.66	24.28
65	100.0	58.0	7.22	0.365	2.66	24.80
	Ca phosphate Precipitate 56-A					
66	5.0	4.35	7.38	1.05	3.01	22.94
67	25.0	21.7	7.29	0.391	2.58	24.55

* The pH was not adjusted during the equilibration.

distributed in liter portions in seven Florence flasks and tightly stoppered. To each of five of the flasks was added a weighed amount of bone powder (previously ground to pass a 100 mesh sieve, washed with water and ether, and dried). The amounts added varied from 1.3 to 100 mg. To one of the remaining flasks 5 mg. and to the other 25 mg. of a calcium phosphate precipitate (No. 56-A) were added. This precipitate was prepared by adding during 4 minutes, with rapid stirring, 94.5 mm of CaCl_2 in 1 liter

to 2 liters of a solution containing 210 mm of Na_2HPO_4 and 30 mm of NaHCO_3 . When the pH had dropped to 7.0, as judged colorimetrically, NaOH (0.4962 N) was added as needed, simultaneously with the CaCl_2 , to maintain the pH at 7.0. The final pH, recorded with the glass electrode, was 6.98. The precipitate was filtered 2 minutes after the completion of the addition of the CaCl_2 , washed with distilled water until free from chloride, dried, and ground to a fine powder. Analysis showed it to contain 8.2 mm of Ca, 0.229 mm of Na, 5.62 mm of PO_4 , and 0.08 mm of CO_2 per gm., which indicates that it consisted essentially of $\text{Ca}_3(\text{PO}_4)_2$. The flasks were rotated continuously for 8 days, at which time the pH was determined electrometrically and a portion of the solution filtered for analysis. The amount of precipitate added was calculated as $\text{Ca}_3(\text{PO}_4)_2$ from the inorganic phosphate content of the bone or precipitate added (Table II). The values of $p[(\text{Ca}^{++})^3 \times (\text{PO}_4^{=})^2]$ obtained in the experiments reported in Tables I and II, as well as others not reported in detail, have been plotted in Fig. 1 as a function of the amount of solid used in the equilibration. It is seen that the ion product, expressed as $p[(\text{Ca}^{++})^3 \times (\text{PO}_4^{=})^2]$, is, as has been previously reported, approximately 27.5 when the solid exceeds 150 mg. per liter. With progressively smaller amounts of solid, the ion product increases and approaches the $p[(\text{Ca}^{++})^3 \times (\text{PO}_4^{=})^2]$ value 23.1 in the presence of 1 mg. of solid.

Comparison of Procedures Used in Determination of Solubility Product—After finding a value for the solubility product of $\text{Ca}_3(\text{PO}_4)_2$ by the first procedure employed, it was our intention to cause the precipitation of varying amounts of calcium phosphate during the equilibration and extrapolate the curve, plotted from such data, to zero precipitate. It would appear evident that if calcium phosphate adsorbs ions from the supernatant fluid, a precipitate formed in the solution would adsorb more ions than would one which had partially come to equilibrium. By forming the precipitate in the solution, one might expect to get the lowest values for the solubility product. Several points were obtained by this procedure. It soon became evident that experimental difficulties, the greatest of which was the task of keeping the pH constant, increased with the amount of precipitate formed. In order that points derived in this manner could represent true values, the pH during equilibration must be the same as that at

which precipitation started. Consequently, the values determined directly with 1 mg. or less of added solid per liter are considered as representing most nearly the true value for the solubility product.

It should be noted that the experiments on the addition of varying amounts of dried calcium phosphate or ground bone to slightly supersaturated solutions are not strictly comparable to those in which the same amount of solid was formed in the solution.

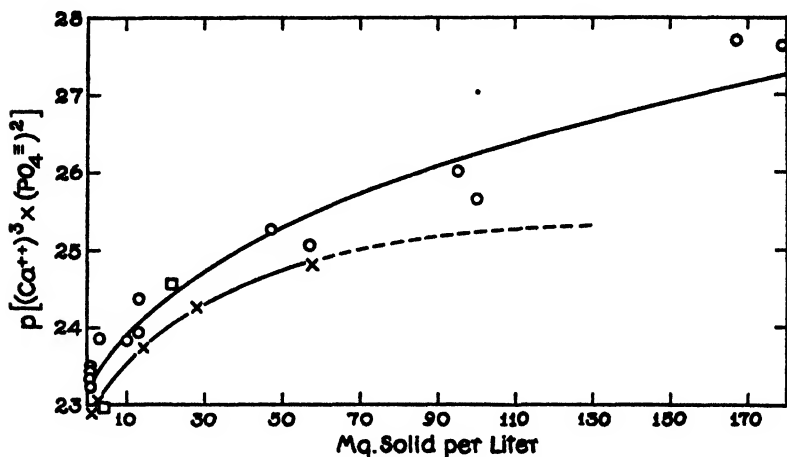


FIG. 1. Ion product $[Ca^{++}]^3 \times [PO_4^{=}]^2$ found by equilibrating for 5 to 10 days with varying amounts of solid. O, precipitate formed in a solution inoculated with less than 1 mg. of calcium phosphate; X, bone powder added to a slightly supersaturated solution (mg. of solid calculated from the bone powder added); □, calcium phosphate added (Precipitate 56-A) to a slightly supersaturated solution (mg. of solid calculated from the precipitate added).

However, with 1 mg. or less of solid per liter, they yield essentially the same results, and with increasing amounts of solid, they both show that the ion product $[Ca^{++}]^3 \times [PO_4^{=}]^2$ decreases. Reasons for expecting quantitative differences in the results when an appreciable amount of precipitate was added or formed are as follows: The extent to which a given amount of precipitate will adsorb ions from a solution is usually qualitatively related to the concentration of the adsorbable ions in the solution, and to the solubility product of the salt formed by that ion and an ion com-

mon to the precipitate (Paneth's rule) (23, 24). It is also obviously related to the amount to which that ion pair has already been adsorbed by the precipitate. Consequently, when a precipitate formed under one set of conditions is transferred to a solution having different ionic concentrations, different ions may be adsorbed. This will depend upon the concentrations of the ions in the solution from which the precipitate was formed, the extent to which the precipitate was allowed to come to equilibrium, and the concentration of the ions in the solution to which it was transferred. Washing the precipitate with water, previous to the transfer, complicates the results, because washing partially removes adsorbed ions. Without an exact understanding of the extent to which individual ion pairs are adsorbed, a prediction of the composition of the precipitate formed under a given set of conditions is not possible. Examples of calcium salts whose ions are present in solution in concentrations close to the solubility product of the salts include CaCO_3 and CaHPO_4 .⁴ The adsorption of any electrically neutral pair of ions, of which one was either calcium or phosphate, would decrease the product $[\text{Ca}^{++}]^3 \times [\text{PO}_4^{=}]^2$.

Ion Products with Large Amounts of Solid Phase—By forming 150 to 200 mg. of solid per liter from solutions containing no bicarbonate (Table I) and equilibrating 7 days, we found the ion product $p[(\text{Ca}^{++})^3 \times (\text{PO}_4^{=})^2] = 27.71$. This corresponds well with the value 27.75 found by Holt, La Mer, and Chown (6) (re-calculated with the same dissociation constants) when they precipitated about 0.9 gm. of calcium phosphate per liter from similar solutions and equilibrated for 8 days. By forming 10 gm. of solid per liter from solutions containing bicarbonate ($\mu = 0.203$) and equilibrating 13 days, we found the ion product $p[(\text{Ca}^{++})^3 \times (\text{PO}_4^{=})^2] = 26.18$ (Table III). This corresponds fairly well with the value 26.36 found by Sendroy and Hastings after 8 days equilibration of a corresponding amount of solid with similar

⁴ The extent to which $\text{Ca}(\text{OH})_2$ is adsorbed has been determined by the experiments of Lorah, Tartar, and Wood (7). Alizarin, of which the calcium salt is fairly insoluble, is strongly adsorbed by the precipitates. Magnesium, which presumably owes its presence in bone salts to its relatively insoluble carbonate and phosphate, is omitted from the discussion for the sake of simplicity. Sodium, although it is the predominant cation of the solution, is but slightly adsorbed by the precipitate.

solutions. The maximum amount of bone employed in our equilibrations (100 mg.) was probably not large enough to indicate the minimum value for the ion product obtainable by equilibration of bone. The values for the ion product were always greater than were found when a corresponding quantity of precipitate was formed in solution. The points fall on a smooth curve which apparently approaches the values for $p[(Ca^{++})^2 \times (PO_4^{=})^2] = 25.5$ to 26.0 which have been found when larger amounts of bone were used in equilibration (16).

The values of the ion products obtained from our experiments with large amounts of solid (Table III) show that the ion products in 4 to 5 hours after precipitation started are below that at which the precipitates could form. The results would indicate that the

TABLE III
Change of Ion Product $[Ca^{++}]^2 \times [PO_4^{=}]^2$ with Time

Experiment No.	Hrs. after pptn.							Days after pptn.		
	0.25	0.5	1.50	1.75	4.00	5.25	24.0	10	13	28
12	22 47			23.54		24.30			26.18	26.61
10		22 62	23.02		24.16		24 97	25 51		

315 mm of $CaCl_2$ in 1 liter of solution were added to 225 mm of Na_2HPO_4 and 30 mm of $NaHCO_3$ in 2 liters of solution. 0.5 M NaOH was added to keep the pH at 7.4 (Experiment 10) and 7.0 (Experiment 12). The solution was constantly stirred at 38° in a closed vessel.

precipitation of tricalcium phosphate is not slower than for other relatively insoluble salts.

Hypotheses Concerning Steps Preceding Precipitations—It has been suggested that the formation of $CaHPO_4$ is a step in the formation of the tertiary phosphate (12, 25). That hypothesis would be difficult to prove or disprove with the evidence available if it were confined to the idea that aggregates of Ca^{++} and HPO_4^{-} ions of less than crystal dimensions are formed. This is because, when the ion products $[Ca^{++}] \times [HPO_4^{-}]$ are calculated from our experiments for solutions equilibrated with minimum amounts of bone or tricalcium phosphate, the results over the pH range 7.0 to 7.6 yield ion products $p[(Ca^{++}) \times (HPO_4^{-})] = 5.59$ to 5.60. These correspond very closely to published values of the

solubility product of CaHPO_4 (pK'_{sp} , 5.60) (12, 26). Assuming that the experiments on which the solubility products of dicalcium phosphate are based are not influenced by a partial conversion to tertiary phosphate, the tendency for the two to form in the physiological range might be considered to be the same. However, the reaction could be expected to go in the direction of the formation of tertiary phosphate without necessarily forming dicalcium phosphate crystals. This would follow because the tertiary phosphate, once formed, adsorbs ions and dissolves only at much lower calcium and phosphate ion concentrations, whereas the CaHPO_4 presumably dissolves at essentially the same concentration as that at which it precipitates. An association of ions corresponding to 3 molecules of dicalcium phosphate might lose to the solution what corresponds to 1 phosphoric acid molecule and tricalcium phosphate would result; or, it might combine with additional Ca^{++} and HPO_4^{-} to form the CaHPO_4 crystal. Whether tricalcium or dicalcium phosphate is obtained as the precipitate would depend on the relative rates of these two processes. The precipitates obtained under the conditions of our experiments did not show crystals of dicalcium phosphate under the microscope, and corresponded closely to the composition $\text{Ca}_3(\text{PO}_4)_2$ when analyzed as quickly as they could be removed from the solution in which they were precipitated (see, for instance, Precipitate 56-A).

When a solution at pH 8.1 was equilibrated with less than 1 mg. of solid per liter, the ion product $p[(\text{Ca}^{++}) \times (\text{HPO}_4^{-})]$ indicated that the solution was undersaturated with respect to CaHPO_4 . The solution contained 0.592 mm of Ca, 1.06 mm of PO_4 per liter which gives $p[(\text{Ca}^{++}) \times (\text{HPO}_4^{-})] = 6.22$ as compared with pK'_{sp} CaHPO_4 5.5 at 25° and $\mu = 0.15$ determined below pH 7.0 (6, 12). No great significance is attached to the comparison of the ion product from our experiment with the determination by others of the solubility product of CaHPO_4 at considerably lower pH. However, the results show that the $p[(\text{Ca}^{++})^3 \times (\text{PO}_4^{-})^2]$ at which precipitation occurs is essentially constant over a wide range of pH. This indicates that the product $[\text{Ca}^{++}]^3 \times [\text{PO}_4^{-}]^2$ is capable of defining the conditions under which the precipitate will form, whether or not the $[\text{PO}_4^{-}]$ ions are the ones primarily involved in the reaction.

Application to Body Fluids—By estimating the calcium ion concentration of serum according to the procedure of McLean and Hastings (27), the $p[(Ca^{++})^3 \times (PO_4^{=})^2]$ has been calculated by Browman (16) and found to be 23.20 for serum as drawn and 23.50 for cerebrospinal fluid. Using data from the literature, we found that $p[(Ca^{++})^3 \times (PO_4^{=})^2]$ varied in normal serum from 23.1 to 23.4. The values which we determined for the $pK'_{Ca_3(PO_4)_2}$, using small amounts of tricalcium phosphate or bone salts, varied between 22.77 and 23.47. The agreement of our determination of the solubility product of these salts with the ion product of blood plasma implies that the blood plasma is probably not supersaturated with respect to the salts of bone, in the sense that bone salts could precipitate from it spontaneously. This conclusion is quite different from that arrived at by equilibration of the blood plasma with relatively large amounts of bone (16) or tertiary calcium phosphate (6, 18). Owing to the fact that adsorption of ions was not taken into account, it was formerly thought that such experiments demonstrated that the blood plasma is supersaturated to the extent of about 200 per cent.⁵

The variations to be expected in the ion product $[Ca^{++}]^3 \times [PO_4^{=}]^2$ of the blood plasma under various conditions and the variations in the experimentally determined values for the solubility product are such that it is not possible to decide whether the plasma is just undersaturated, saturated, or may not, at times, be slightly supersaturated. The agreement between the two values is sufficiently close, however, to indicate that if calcification is to be initiated and proceed at a rate, such as occurs in growth, an increase of the ion product at the site of calcification is a necessity.

Once formed, the bone salts could not be dissolved unless the concentrations of the ions composing them were decreased locally to values considerably lower than those existing in the blood plasma. The growth of bone is characterized by the solution of the bone salts in one part of the structure and the simultaneous

⁵ When the blood plasma is shaken for 30 minutes with reagent grade tricalcium phosphate, the solid takes up $CaCO_3$ below the solubility product of that salt (18), but the phosphate concentration of the plasma is not decreased. The phenomenon, for which an explanation has not previously been offered, can be explained by the adsorption of the Ca^{++} and $CO_3^{=}$ ions by the precipitate.

deposition at another. Experiments in which large amounts of bone salts were equilibrated with relatively small amounts of solution have, therefore, helped to define the conditions under which the solution may take place, but are not applicable to conditions affecting the precipitation.

From the experiments herein reported, the conclusion seems unavoidable that the metabolic activity of cells is a prerequisite both for the precipitation and the solution of the calcium salt of bone.

SUMMARY

The ion product $[Ca^{++}]^3 \times [PO_4^{=}]^2$ increases as the amount of bone or tricalcium phosphate, equilibrated with solutions of their ions, decreases below 150 mg. per liter. The solubility product of this substance, expressed as $p[(Ca^{++})^3 \times (PO_4^{=})^2]$, is 23.1 (± 0.4).

The chemical natures of the calcium salts of bone and the conditions necessary for their formation are discussed. Evidence is presented for the conclusion that the bone salts cannot precipitate spontaneously from the blood plasma unless the ion product is increased. Once formed, the bone salts cannot dissolve unless the concentrations of the ions composing them are decreased below the concentrations found in the blood plasma.

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THE GLUTAMIC ACID-PYRROLIDONECARBOXYLIC ACID SYSTEM

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The investigation of this reaction was originally undertaken with the object of determining the extent to which it might interfere in the routine procedures of protein analysis or might be exploited as a method of analysis. The results obtained raised a number of interesting theoretical problems. These have been examined in some detail and are now submitted as a contribution to the problem of the formation and rupture of the amide linkage.

We owe to Abderhalden and Krauttsch (1), Foreman (2), Skola (3), Okinaka (4), and Bethke and Steenbock (5) our knowledge of the behavior of this system in dilute aqueous solution. These observers have shown that the system is a reversible one in which true equilibria may be attained under favorable experimental conditions, and that the equilibria and the rates at which they are approached are very sensitive to the acidity or alkalinity of the solution. In solutions of strong acids or bases, and at temperatures approaching 100°, equilibrium is attained in a few hours and is such that glutamic acid is the predominant component. In solutions of weak acids or bases, the rates of reaction are less rapid, and equilibrium has shifted in favor of extensive anhydride formation. In the region of neutrality rates of change are so slow, even above 100°, that equilibrium conditions have not been established. We may conclude that the important variable, apart from temperature, is the hydrogen ion concentration. Unfortunately the data are inadequate for a comprehensive definition of the influence of this variable. For this reason, we have undertaken further observations.

EXPERIMENTAL

Equilibria—A series of reaction mixtures was prepared by addition of hydrochloric acid or of sodium hydroxide to standard solutions of glutamic acid or of pyrrolidonecarboxylic acid. The amounts of acid or of alkali added were chosen to give mixtures covering the whole range of pH over which the reactants themselves or the added acid or alkali were effective buffers. Samples of each mixture were sealed in a series of glass tubes and immersed in a freely boiling bath of alcohol (78°), water (100°), or acetic acid (118°). Tubes were removed at intervals and determinations of amino nitrogen were made by the manometric method of Van Slyke. When successive determinations showed that equilibrium had been attained, the pH of the equilibrium mixture was determined with the aid of the hydrogen electrode at 25°. From the amino nitrogen determinations and the total reactant concentrations we calculated $K = [\text{anhydride}]/[\text{glutamic acid}]$. The results will be found in Table I.

Velocities of Reaction—The ionic equilibria of the reactants are such that the progress of the reaction is attended by considerable changes in pH. Since the velocities are, themselves, a rather complex function of pH, it is possible to obtain a simple order of reaction only if the pH be stabilized by the presence of an excess of a foreign buffer system. Under these conditions the reaction was found in all cases to follow the equation for a reversible first order process

$$t(k' + k'') = \ln \frac{Ka}{Ka - (K + 1)x}$$

where the velocity of dehydration = $k'[\text{glutamic acid}]$ and the velocity of hydration = $k''[\text{anhydride}]$, a and x having their customary significance.

A reaction mixture of the desired pH was prepared by the addition of a suitable buffer to a solution of one or other of the reactants. Samples were sealed in a series of tubes and were immersed in a bath at the chosen temperature. At measured intervals of time a sample was removed and the amino nitrogen determined. Observations were continued, where possible, until equilibrium was attained in order to obtain an experimental value

TABLE I
Equilibria

Temperature	pH ₂₅	pH ₁₀₀	μ	Anhydride	Log <i>K</i>	Log $\frac{a_2}{a_1}$	Log $\frac{b_2}{b_1}$	Log $\frac{c_2}{c_1}$
°C.				per cent				
100	-0.28		2.0	1.85	-1.72	(3.0)		
	+0.13		0.75	8.0	-1.06	(2.8)		
	0.34		0.48	10.05	-0.95	2.86		
	0.52		0.34	14.7	-0.73	2.79		
	0.78		0.18	26.5	-0.465	2.84		
	0.98		0.13	35.0	-0.270	2.86	2.84	
	0.99		0.13	37.2	-0.227	2.83		
	1.30		0.07	54.2	+0.072	2.85		
	1.55		0.043	66.7	0.301	2.86		
	1.73	1.75	0.023	72.5	0.423	2.92		
	1.81	1.84	0.028	76.0	0.501	2.91		
	2.09	2.20	0.016	85.4	0.74	2.94		
	2.36	2.60	0.010	88.3	0.88	3.01	4.31	
	2.44	2.69	0.010	89.3	0.92	3.02	4.32	
	2.70	2.98	0.011	92.2	1.07	2.99	4.29	
	3.11	3.39	0.020	94.0	1.20	3.01	4.31	
	3.96	4.23	0.045	97.8	1.65	3.02	4.31	
	11.81	10.11	0.021	62.7	0.220	3.00		3.47
	11.84	10.14	0.085	53.5	0.100	2.99		3.46
	12.14	10.44	0.027	43.2	-0.094	2.97		3.44
	12.72	11.02	0.060	14.3	-0.775	3.01		3.48
	12.75	11.05	0.140	9.2	-0.917	3.01		3.48
	13.38	11.68	0.290	1.9	-1.58	3.03		3.48
		pH ₇₈						
78	0.98		0.13	28.5	-0.400	2.99		
	2.51	2.62	0.01	86.3	+0.804		4.30	
	12.11	10.79	0.027	37.1	-0.231			3.57
		pH ₁₁₈						
118	0.78		0.18	29.8	-0.372	2.75		
	1.715		0.023	76.8	+0.519	2.81		
	2.38	2.79	0.010	91.8	1.049		4.35	
	11.87	9.91	0.021	66.5	0.296			3.34
	12.17	10.21	0.027	50.3	0.004			3.32
	12.73	10.77	0.060	18.8	-0.638			3.37

for K . An occasional determination of pH was made to check the efficiency of the buffer. The above equation was, then, employed to calculate k' and k'' (natural logarithms, time measured in seconds). The results will be found in Table II.

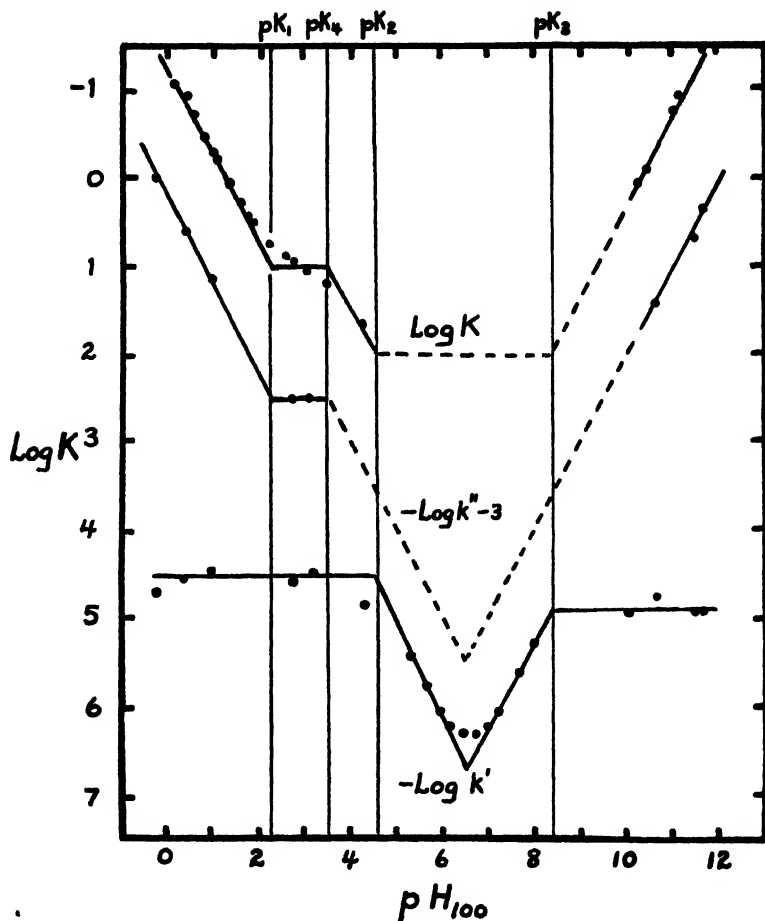


FIG. 1. Relation between K (equilibrium constant), k' (velocity constant of dehydration), k'' (velocity constant of hydration), and pH.

In the region of neutrality the rates of reaction were so slow that equilibrium was not approached after many days. Under these conditions, however, solutions of glutamic acid did react significantly, yielding good monomolecular constants (k'). Solu-

tions of pyrrolidonecarboxylic acid, on the other hand, exhibited no measurable formation of amino nitrogen in periods up to a week. Determinations of K and k'' were, therefore, not possible. It is evident, however, that K cannot be much less than 100 and that k'' is correspondingly smaller than k' .

Results—The nature of the relations between pH and K , k' , and k'' at 100° may be most briefly presented diagrammatically. This is done in Fig. 1. The broken lines are plausible extrapolations of the curves into regions of pH in which direct observations have not been possible. The calculation of pH_{100} is explained below. Certain conclusions, touching the problem of protein analysis, may be drawn.

1. In solutions close to neutrality equilibrium favors almost complete dehydration of glutamic acid. The rate of change, however, is so slow that only about 1 per cent conversion occurs in 2 to 3 hours at 100°. In protein analyses involving distillation of neutral hydrolysates there is no danger of loss of glutamic acid, provided the solution is not submitted for long periods to temperatures approaching 100°.

2. In strongly acid or alkaline solution (not less than 2 M hydrochloric acid or 0.5 M sodium hydroxide) the conversion of pyrrolidonecarboxylic acid to glutamic acid is rapid and practically complete. There is, therefore, no danger of dehydration of glutamic acid during the acid or alkaline hydrolysis of protein if the concentrations of acid or of alkali in excess of that bound by the amino acids are not less than those quoted.

3. We were interested in the possibility of using this reversible change for the isolation and determination of glutamic acid. The choice of conditions for converting glutamic acid to pyrrolidonecarboxylic acid involves a compromise between favorable rates and favorable equilibria. At pH 4 or 10 the reaction proceeds to about 98 per cent completion in somewhat less than 50 hours at 100°. Between pH 2 to 3 the rate is about twice as rapid but equilibrium corresponds to only 90 to 94 per cent pyrrolidonecarboxylic acid. At 120° and pH 3, the time is reduced to less than 3 hours and the equilibrium is slightly more favorable than at 100°. If this reaction is to be undertaken, we suggest the solution be adjusted to pH 4 and heated for 50 hours at 100° or for 6 hours at 120°. In unbuffered solutions the pH will fall

to about 3. In a protein hydrolysate, the other amino acids present will diminish this change.

4. The best conditions for the hydrolysis of the anhydride are 1 to 2 hours at 100° in not less than 2 M hydrochloric acid or 0.5 M sodium hydroxide.

A few preliminary experiments on hydrolysates of proteins may be mentioned. Protein hydrolysates were boiled for several days at 100° after adjustment to pH 4. Periodic determinations of amino nitrogen were made. The fall in amino nitrogen continued long after dehydration of glutamic acid should have been completed and far exceeded the amount of this amino acid present. As was to be expected, extensive anhydride formation by other amino acids was occurring.

Solutions of the barium salts of the dicarboxylic acids were prepared from a hydrolyzed protein by the method of Foreman. These solutions were adjusted to pH 4 and heated at 100° for several days. The curve of amino nitrogen did not fall to a precise limiting value, though it was not difficult to extrapolate it to a plausible value for the glutamic acid originally present. The precipitate obtained by Foreman's method is far from a pure mixture of dicarboxylic acids. When this precipitate was dissolved and reprecipitated by alcohol, it gave, under the above conditions, a curve very similar to that for glutamic acid. The results were, however, lower than established values for the glutamic acid contents of the proteins used.

Attempts to separate glutamic acid as the anhydride were made. Protein hydrolysates were submitted to the conditions described, then concentrated to a small volume, and acidified to about pH 1. This solution was continuously extracted with butyl alcohol or with ethyl acetate. The former solvent rapidly extracted all the pyrrolidonecarboxylic acid but also removed much other nitrogen. The ethyl acetate was much more specific but we have not found convenient conditions for complete extraction. The pyrrolidonecarboxylic acid was recovered from the extracts by shaking out with alkali. By boiling the aqueous extract for 2 hours in concentrated hydrochloric acid, concentrating, and saturating with hydrochloric acid gas, glutamic acid hydrochloride was obtained in the usual manner. Our results on egg albumin, edestin, and casein have all been somewhat lower than

the most dependable values in the literature. These experiments do not encourage the hope of developing a simple method for the separation or determination of glutamic acid as its anhydride such as would be a useful routine in protein analysis. It is possible, however, that this reaction may find a use in special cases such as the separation of glutamic and hydroxyglutamic acids from one another. It is known that, under comparable conditions, the latter acid is dehydrated much more rapidly than is glutamic acid (6).

Dissociation Constants—Acceptable values for the dissociation constants of the reactants and approximations to the activity coefficients of their ions at experimental ionic strengths were necessary for a theoretical analysis of our results. There are discrepancies in the various recorded values of the dissociation constants of glutamic acid. We are aware of no previous determination of the constant of pyrrolidonecarboxylic acid. We, therefore, determined these constants both in pure solutions of various strengths and in solutions in which the ionic strengths were varied by addition of potassium chloride. After the completion of this work, a report on the constants of glutamic acid and its esters in potassium chloride solutions was published by Neuberger (7). Our results are in very satisfactory agreement with his and so details may be omitted in submitting our values. The constants in the Debye-Hückel equation which we use to define the activity coefficients are not identical with his but the effects of the discrepancies are small.

The method of discontinuous titration at the hydrogen electrode was employed, with a battery of Clark rocking half-cells and a saturated calomel electrode. The latter was standardized by 0.01 M hydrochloric acid in 0.09 M potassium chloride for which a pH of 2.080 was assumed. This corresponds, in our experience, with a value of 1.098 for 0.1 M hydrochloric acid, making no allowance for liquid junction potentials.

The glutamic acid was a carefully purified preparation from a commercial product. The pyrrolidonecarboxylic acid was prepared from this by boiling in aqueous solution until equilibrium had been obtained. The solution was evaporated to dryness and extracted with alcohol. The pyrrolidonecarboxylic acid was, then, repeatedly recrystallized from aqueous alcohol until

free from significant amounts of amino nitrogen. The reactants were titrated in 0.01 to 0.04 M concentration. The potassium chloride was varied from 0 to 0.25 M. The calculation of apparent constants (K') followed established practise. For the calculation of the two overlapping constants of glutamic acid we employed the method of Simms (8). We obtained $[H^+]$ and $[OH^-]$ from pH with the aid of experimental curves for the pH of solutions of hydrochloric acid and sodium hydroxide (0.01 to 0.1 M) in potassium chloride solutions (0 to 0.25 M). Extrapolation of the $pK'_w: \sqrt{\mu}$ curves gave $pK_w = 13.93$. This has been used in our calculations.

From the curves relating apparent constants to $\sqrt{\mu}$ we derived true constants (K) and approximate activity coefficients on the following assumptions.

1. $K = K'\gamma_B/\gamma_A$ where γ_B and γ_A are the activity coefficients of the conjugate base and conjugate acid, respectively.

2. The activity coefficient of neutral molecules (whether uncharged or dipolar ions) was unity in all our experimental systems.

3. $-\log \gamma = (A\sqrt{\mu})/(1 + B\sqrt{\mu})$, where $A = 0.5$ for a monovalent ion (including the monoanion of glutamic acid which also possesses a dipolar ion) and $A = 2$ for a bivalent ion. B was treated as an empirical constant, whose values were to be assessed from the $pK': \sqrt{\mu}$ curves.

The two latter assumptions, though only approximations, may be expected to yield coefficients of the right order of magnitude for the majority of our reaction mixtures. Applying them to the $pK': \sqrt{\mu}$ curves, we have derived the following results.

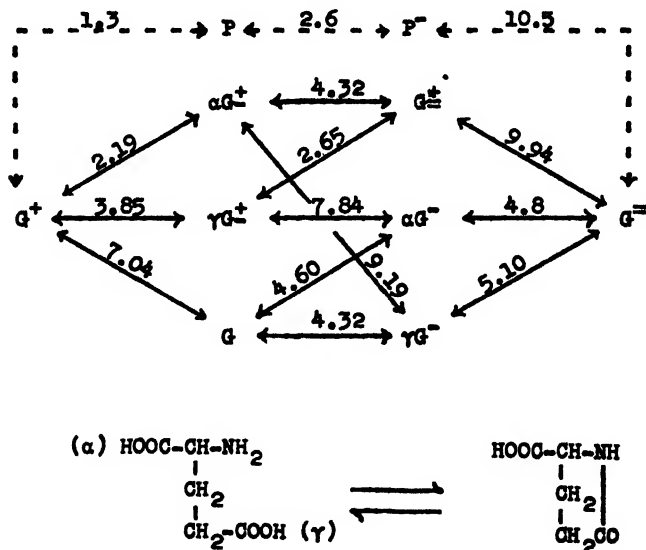
Dissociation Constants—Glutamic acid $pK_1 = 2.19$; $pK_2 = 4.32$; $pK_3 = 9.94$; pyrrolidonecarboxylic acid $pK_4 = 3.32$.

Activity Coefficients—For the cation and monoanion of glutamic acid and the anion of pyrrolidonecarboxylic acid we find $-\log \gamma = 0.5\sqrt{\mu}/(1 + 1.5\sqrt{\mu})$. For the bianion of glutamic acid $-\log \gamma = 2\sqrt{\mu}/(1 + 2\sqrt{\mu})$. The magnitudes of the B constant are reasonable but, in so far as they bear the brunt of the approximations, have no precise physical significance.

Ionic Forms of the Reactants—In our discussion of this system it will be necessary to distinguish the individual ionic forms of the reactants. P and P^- will represent the unionized form and the anion, respectively, of pyrrolidonecarboxylic acid. G^+ ,

αG^{\pm} , G^{\pm} , and G^{-} will represent the four forms of glutamic acid which successively predominate in solution as the pH is raised. These are the four molecular species which are related by the three dissociation constants given above. The Greek prefix to one of these symbols indicates the position of the negative charge with respect to the NH_3 group.

In addition to these four forms of glutamic acid two other neutral forms and two other monoanions may occur. Although these do not contribute significantly to the ionic equilibria, they



Pyrrolidonecarboxylic Acid

Glutamic Acid

FIG. 2. Ionic equilibria of glutamic acid

must be considered here because they may occur in sufficient concentration to have kinetic importance. These additional forms are related to each other and to the predominating molecular species by 9 additional hydrogen ion dissociation constants. The situation is illustrated in Fig. 2. The dotted chain of equilibria concern the reversible formation of pyrrolidonecarboxylic acid and will be considered later. Each arrow represents a single proton transfer. The numbers above the arrows are the hypothetical pK values of these transfers in water. Some of these

are given by Neuberger and are derived from his observations on the esters of glutamic acid by arguments discussed by him. The others we have derived by similar arguments from Neuberger's data.

Temperature Coefficients of Ionization—It has been necessary to determine the velocity and equilibrium constants of this system at elevated temperatures. The ionic equilibria, on the other hand, have been determined from pH and pK measurements at 25°. A theoretical treatment of the results requires a correlation of these two sets of data. A direct combination of them is legitimate in some cases if the pH of the system is determined solely by the ionization of the reactants themselves. The ionization of any added buffer, including strong acid or alkali, is likely to be affected by temperature to a different degree than the particular ionizations of the reactants which control velocities. In that case true values for intrinsic velocity or equilibrium constants will not be obtained by combination of data at different temperatures. Moreover, heats of reactants and critical increments may be seriously modified.

It is not possible to make any close estimate of the corrections which should be applied to the results here reported, because the necessary temperature coefficients have not been measured. A recent paper by Harned and Embree (9) does, however, encourage an attempt to estimate the order of magnitude of the effect. These authors have correlated the changes with temperature of the pK values of a number of weak acids and of two amino acids—glycine and alanine. They find that the behavior of all these constants may be described by an empirical equation involving two individual constants characteristic of the particular electrolyte. These constants are (a) the maximum ionization constant (k_m) of the acid or base, and (b) the temperature, θ , at which this maximum occurs. Certain regularities are observed in θ as a function of k_m . They have led us to make the following assumptions: (1) K_3 and K_4 have the same θ as simple aliphatic carboxylic acids, specifically, acetic acid; (2) K_1 has the same θ as K_2 of alanine; (3) K_w/K_3 has the same θ as k_b of alanine.

Using these values for θ , we have calculated the values of our dissociation constants at the three temperatures at which we have conducted our studies. These calculated values are given

in Table III. The second constant of phosphoric acid is included because we have used this buffer in some experiments. Its temperature coefficient was measured by Nims (10). The values for K_2 are interpolations from those given in "International critical tables" adjusted to conform to our experimental value at 25°.

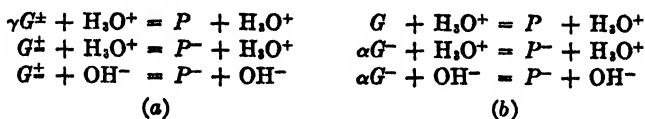
Mechanism of Reaction—Fig. 1 has been drawn to emphasize the way in which the curves relating equilibria and velocities to pH are oriented about the dissociation constants of the reactants. The curves are consistent with a number of alternatives. In our choice between these we have been influenced by the following considerations: (1) A reversible bimolecular process involving reactants and solvent is, kinetically, the most plausible. (2) The charged or uncharged condition of the two groups of glutamic acid which condense to the amide group will fundamentally modify reaction rates. It may be anticipated that a particular distribution of charges on these groups will uniquely favor reaction. It follows that a reaction mechanism which assigns kinetic significance to a particular ion of glutamic acid must extend kinetic significance to all other ions with the same distribution of charges on the condensing groups. (3) The ionization of the αCOOH of glutamic acid and of the corresponding group in pyrrolidonecarboxylic acid may be expected to influence reaction rates only to a secondary degree, reflecting differences in the electrostatic work between the colliding molecules.

The validity of these considerations is illustrated in Fig. 1 where it is seen that K_2 and K_3 alone determine the inflections of the curve for k' . No influence of K_1 is betrayed. In the k'' curve, moreover, K_4 only appears as a minor displacement.

The $\log k''$:pH curve, obviously, suggests a reaction involving either P or P^- and either H^+ or OH^- . There seems to be no alternative, unless we assign to the amide group weak acidic and basic properties. This, indeed, is plausible but is not a profitable basis for a quantitative analysis of the system, since we are unable to measure the extents of these hypothetical ionizations. If we accept the simpler interpretation of the curve, then the inflection observed close to pH 6 corresponds with the pH at which $P^- + \text{H}^+$ and $P^- + \text{OH}^-$ are equal in rate. The small displacement in the curve as the pH falls from 4 to 2 indicates the region in which control of the observed velocity passes from $P^- + \text{H}^+$ to $P + \text{H}^+$.

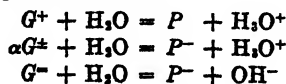
The log k' :pH curve offers several alternative interpretations. It is consistent with a reaction catalyzed by H^+ or OH^- . In this case the active forms of glutamic acid would have to be the neutral molecule and the monoanion. The situation is, however, complicated by the existence of each of these species in three tautomeric forms. The neutral molecule may be αG^\pm , γG^\pm , or G and the anion may be G^\pm , αG^- , γG^- . These alternatives may be reduced if we apply the principle that the active reactants shall have a common distribution of charges on the condensing groups. There are, then, two possibilities. These groups (a) may be uncharged or (b) may constitute a dipolar ion pair. In the former case the reactants would be G and αG^- ; in the latter γG^\pm and G^\pm .

We have System I, a catalytic mechanism involving (a) a prototropic change and (b) no proton shift.



A simpler situation results if we assume that glutamic acid reacts with H_2O rather than with its ions. Three active forms—the cation, the neutral molecule, and the bianion—are, then, necessary to satisfy the curve. The neutral molecule has three tautomeric forms. Since, however, the cation is one reactant in acid solution, we may assume that the neutral molecule, which also is an effective reactant in acid solution, must likewise have a charge on the NH_2 and none on the $\gamma COOH$ group. This identifies the neutral reactant with αG^\pm .

System II, a single proton transfer.



Expressed as equilibria, Systems I and II are only formal alternatives. On the basis of the kinetic theory of reaction velocity it is possible, however, that one set of reactions will prove more plausible than the other. In some degree, System I really avoids the issue of mechanism by assuming a catalytic

process. System II is to be preferred on this count. The latter has the added advantage that the reactive forms of glutamic acid which it postulates are the ions whose concentrations are determined by the conventional dissociation constants of glutamic acid. For System II, we have

$$\begin{aligned} k'[\text{G.A.}] &= h_2o[a_1(G^+) + b_1(\alpha G^\pm) + c_1(G^-)] \\ \text{and} \quad k''[\text{P.C.A.}] &= a_2 \cdot h(P) + b_2 \cdot h(P^-) + c_2 \cdot oh \cdot (P^-) \end{aligned}$$

where [] and () represent concentrations and activities respectively, h_2o , h , and oh represent (H_2O) , (H^+) , and (OH^-) respectively, a_1 , $b_1 \dots b_2$, c_2 are intrinsic velocity constants defined by the equations, and G.A. and P.C.A. represent glutamic acid and pyrrolidonecarboxylic acid respectively.

Applying the mass law to the various ionic equilibria, in the usual manner, we derive

$$k' = \frac{h_2o}{z} [a_1 h^3 + b_1 K_1 h^3 + c_1 K_1 K_2 K_3] \quad (1)$$

$$\text{and} \quad k'' = \frac{1}{y} [a_2 h^3 + b_2 K_4 h^3 + c_2 K_4 K_w] \quad (2)$$

$$\text{where} \quad z = \frac{h^3}{\gamma_1} + K_1 h^3 + \frac{K_1 K_2 h}{\gamma_1} + \frac{K_1 K_2 K_3}{\gamma_2}$$

$$\text{and} \quad y = \frac{K_4 h}{\gamma_1} + h^3$$

γ_1 being the activity coefficient of either G^+ , G^\pm , or P^- and γ_2 being that of G^- .

Finally, since the equilibrium state comprises three simultaneous equilibria, we have

$$\frac{[\text{P.C.A.}]}{[\text{G.A.}]} = K = \frac{a_1 h_2o \cdot (G^+)}{a_2 h (P)} = \frac{b_1 h_2o (\alpha G^\pm)}{b_2 h \cdot (P^-)} = \frac{c_1 \cdot h_2o \cdot (G^-)}{c_2 \cdot oh \cdot (P^-)}$$

which gives

$$\frac{Kz}{y \cdot h_2o} = \frac{a_1}{a_2} = \frac{K_1 b_1}{K_4 b_2} = \frac{K_1 K_2 c_1}{K_4 K_3 c_2} \quad (3)$$

where $K_3 = K_w/K_4$.

Under suitable experimental conditions Equations 1 and 2 may be simplified in such a way as to permit the determination of a single intrinsic constant.

1. In solutions containing an excess of hydrochloric acid (below pH 1) our theory makes a_1 and a_2 the rate-controlling constants. Equations 1, 2, and 3 reduce to

$$k' = a_1 \cdot h_2 o \cdot \gamma_1, \quad k'' = a_2 \cdot h, \quad \text{and} \quad K = \frac{a_1 \cdot h_2 o \cdot \gamma_1}{a_2 \cdot h} \quad (4)$$

2. In solutions containing excess of sodium hydroxide (above pH 11) the significant constants are c_1 and c_2 . We then have

$$k' = c_1 \cdot h_2 o \cdot \gamma_2, \quad k'' = c_2 \cdot o h \cdot \gamma_1, \quad \text{and} \quad K = \frac{c_1 \cdot h_2 o \cdot \gamma_2}{c_2 \cdot o h \cdot \gamma_1} \quad (5)$$

We will assume that the activity coefficients do not change with temperature. We may, also, assume that h in Equation 4 and oh in Equation 5 are independent of temperature. These equations have been applied to appropriate experimental values of k' and k'' to derive values for a_1 , a_2 , c_1 , and c_2 with the results shown in Table II.

3. Fig. 1 suggests that k' does not vary much below pH 4. We determined the rate of dehydration of a solution of free glutamic acid (0.05 M). During the progress of the reaction the pH fell from 3.25 to 2.44. In spite of this, the reaction followed a monomolecular course. In this region of pH, Equation 1 closely approximates $k' = h_2 o / K_1(a_1 h + b_1 K_1)$. Since k' was found to be independent of pH, the term $a_1 h$ is negligible and $k' = b_1 \cdot h_2 o$. From this we derive $\log b_1 = -6.26$. A determination of k' was made in 0.5 M citrate solution at pH 2.89. The same equation applies, giving $\log b_1 = -6.19$.

Under the special conditions which have been outlined, all of the intrinsic velocity constants, except b_2 , have been evaluated. From an independent series of equilibrium determinations (Table I) we have also evaluated a_1/a_2 (from data below pH 1) and c_1/c_2 (from data above pH 11). All of these calculations are independent of effects of temperature upon ionization. The following may be taken as the mean values at 100°.

$\log a_1 = -6.06$	$\log b_1 = -6.26$	$\log c_1 = -6.17$
" $a_2 = -3.22$	(one determination)	" $c_2 = -2.74$
" $\frac{a_1}{a_2} = -2.84$		" $\frac{c_1}{c_2} = -3.48$

The equilibrium constants are in satisfactory agreement with the independent determinations of the component velocity constants.

It is not possible to confirm b_1 from the experiments in the acetate and phosphate buffers or to evaluate b_2 and b_1/b_2 from appropriate experiments without introducing a temperature cor-

TABLE II
Velocity Constants

	Temper- ature	pH ₂₅	pH _{temp.}	μ	-Log k'	-Log k''	-Log a_1	-Log b_1	-Log c_1	-Log c_2	-Log b_2	-Log c_3
	°C.											
HCl buffer	100	-0.28		2.0	(4.71)	3.00	(6.25)					
		+0.34		0.48	4.53	3.58	6.09			3.28		
		0.98		0.13	4.41	4.14	6.03			3.24		
Unbuffered		2.44	2.69	0.015	4.58	5.50		6.26			1.98	
Citrate		2.89	3.17	0.25	4.45	5.45		6.19			1.95	
Acetate		3.96	4.24	0.03	4.84			6.29				
		4.96	5.24	0.06	5.43			6.26				
		5.33	5.61	0.07	5.76			6.28				
		5.60	5.88	0.08	6.03			6.30				
Phosphate		6.31	6.40	0.072	6.28			6.24	6.14			
		6.50	6.65	0.090	6.29				6.18			
		6.79	6.94	0.105	6.20				6.23			
		7.00	7.15	0.11	6.04				6.22			
		7.43	7.58	0.123	5.60				6.16			
		7.75	7.90	0.14	5.24				6.17			
Carbonate		9.99		0.14	4.93				6.19			
NaOH		12.24	0.027	4.70	4.50				6.19			2.74
		13.13	0.20	4.92	3.67				6.17			2.74
		13.38	0.29	4.94	3.34				6.14			2.66
HCl buffer	78	0.98	0.13	5.21	4.81	6.83				3.83		
Unbuffered		2.51	2.62	0.015	5.57	6.37		7.22			2.93	
Carbonate		9.99	0.14	5.72					6.97			
NaOH		12.24	0.027	5.47	5.12				6.96			3.37
		13.13	0.20	5.72	4.32				6.96			3.39
Citrate	118	2.89	3.33	0.25	3.70	4.86		5.44			1.20	

rection. We have made this correction by calculating the pH at the experimental temperature. In buffered systems, the change in pH with temperature was assumed to be that of the buffer (Table III). In unbuffered systems the corrected pH was calculated from pH₂₅, K , and the pK values at the experi-

mental temperatures. When the equations were applied to the corrected data, these pK values were also used.

The determinations of k' in acetate and in phosphate buffers were, then, used to calculate b_1 and c_1 . In this region of pH, Equation 1 reduces to

$$k' = \frac{h_2 o}{z} (b_1 K_1 h^2 + c_1 K_1 K_2 K_3)$$

The minimum velocity is close to pH 6.3. On the acid side of this, b_1 plays the chief part in controlling k' . If we assume that $\log c_1 = -6.17$, we may calculate b_1 from observations in this region. Above pH 6.3, the term involving c_1 is the predominant one. Assuming $\log b_1 = -6.26$, we may obtain c_1 from observations of k' under these conditions. The results of these calculations are given in Table II. They confirm the values found in the experiments already reported.

A direct evaluation of b_2 from velocity studies is difficult. In the region of pH in which b_2 controls k'' , equilibria are unfavorable for a direct determination of the latter. We must be content to derive b_2 from b_1 and b_1/b_2 . The region in which b_1/b_2 directly controls equilibria is between pH 2 and 4. In Table I we report a consistent group of values for this constant calculated from observations of K in this range. As a mean value we adopt $\log b_1/b_2 = -4.31$. Then, if $\log b_1 = -6.26$, we have $\log b_2 = -1.95$.

All of the hypothetical constants have now been evaluated. In each case we have used data relating to systems in which the constant concerned was the prevailing one. The validity of Equation 3, which permits the calculation of the equilibrium constants from K at any pH, remains to be tested. This we have done by calculating a_1/a_2 from all equilibrium determinations at 100° (Table I).

DISCUSSION

The experiments which have been described were designed to give a broad description of the system over the widest practicable range of pH. Analysis of the results at 100° in terms of System II has been successful in describing the effects of pH on velocities and on equilibria. On the other hand, Equation 3 does not apply

with precision. The value of $\log a_1/a_2$ shows a distinct change as pH rises from 1 to 3. It would be reasonable to suspect an error in the temperature corrections of K_1 . A much more refined control of the variables—activity coefficients, liquid junction potentials, temperature corrections, and specific catalytic effects—will be necessary to resolve this anomaly. In the meantime the following relationship is significant. We find $\log a_2 = -3.22 = \log b_2 - 1.27 = \log c_2 - 0.48$. Now, from Table III, $\log K_4/K_1 = -1.28$ and $\log K_4K_B/K_1K_2 = -0.45$. We conclude that the empirical relation

$$a_2 = \frac{K_4 b_2}{K_1} = \frac{K_4 K_B c_2}{K_1 K_2} \quad (6)$$

holds with some precision. This can be reconciled with Equation 3 only if $a_1 = b_1 = c_1$. The discrepancies attending the application of Equation 3 are, therefore, dependent upon the differences found between the values of the three latter velocity constants. The data available do not permit us to decide whether these differences are significant or not. If they are real, then we are inclined to attribute them to specific local effects superimposed on the general operation of System II. In the meantime we feel entitled to accept the approximation $\log a_1 = \log b_1 = \log c_1 = -6.22$. Then the corrected values for the equilibrium constants will be $\log a_1/a_2 = -3.00$, $\log b_1/b_2 = -4.27$, and $\log c_1/c_2 = -3.48$. These constants are, by hypothesis, simply proton affinity constants. We may redefine them in terms comparable with acid-base dissociation constants. At equilibrium

$$a_1 \cdot h_2 o(G^+) = a_2 \cdot h(P)$$

$$i. e. \quad \frac{a_1 \cdot h_2 o}{a_2} = \frac{(P)h}{(G^+)} = A \quad \text{and} \quad pA = 1.3$$

$$\text{Similarly} \quad \frac{b_1 \cdot h_2 o}{b_2} = \frac{(P^-)h}{(\alpha G^+)} = B \quad \text{and} \quad pB = 2.6$$

$$\text{also} \quad \frac{c_2}{c_1 h_2 o} = \frac{(G^-)}{(P^-)oh} \quad \text{or} \quad \frac{c_2 k_w}{c_1 \cdot h_2 o} = \frac{(G^-)h}{(P^-)} = C \quad \text{and} \quad pC = 10.5$$

These constants have been inserted in Fig. 2 to illustrate their relation to the other acid-base constants. They are not quanti-

tatively comparable, because they refer to 100° , whereas the pK values are for 25° . The intent of our argument is that the reactions by which glutamic acid and pyrrolidonecarboxylic acid are converted into one another behave as though they were merely slow ionizations. Indeed A may be regarded as a Bronsted constant for the basic properties of the amide group in uncharged pyrrolidonecarboxylic acid. The corresponding constant when pyrrolidonecarboxylic acid is negatively charged is B , and C is the constant for the acid properties in the negatively charged molecule.

The way in which these constants are modified by molecular and electrostatic forces is given by Equation 3. It is significant that these effects are entirely due to influences operative on one of the component velocities (Equation 6). The substantial equality of a_1 , b_1 , and c_1 shows that the rate of reaction of water with glutamic acid is unaffected by the charge on the latter reactant. This presumably reflects the absence of electrostatic forces between the neutral water molecule and the various ions of glutamic acid. Equation 6, on the other hand, illustrates the effect of these forces on reaction rates when both reactants carry a charge. The ratio K_4/K_1 , ostensibly, gives the effect of opening the anhydride ring on the ionization of the α -carboxyl group. We find that it defines the effect of a negative charge at the α -carboxyl on the rate of opening the ring. Again K_B/K_2 represents the relative acid-base affinities of the groups which condense to form the amide group. It is equal to K_w/BC , which we interpret as the ratio of the acidic and basic affinities of the group itself. The rates at which P^- reacts with H_3O^+ and OH^- , respectively, are proportional to this ratio. It is interesting to note that, in this case, H_3O^+ reacts about 8 times as rapidly as OH^- . If, however, we compare $P + H_3O^+$ with $P^- + OH^-$ (a_2 and c_2), we find OH^- is about 2.5 times as effective as the hydrogen ion.

In conclusion we may remark that certain of the relationships which have been discussed might profitably have been developed from Bronsted's extended theory of acid-base catalysis. We have not, however, found this approach as comprehensive as that which we have adopted.

Heats of Reaction; Critical Increments—Data in Tables I and

II may be used to calculate ΔH for K and E for k' and k'' . The values so obtained have little significance. They will include heats of ionization to an extent dependent upon the pH of observation. More useful results are obtained if the van't Hoff and Arrhenius equations are applied to the intrinsic constants. In doing this we have chosen pairs of observations at the same ionic strength and comparable pH but at two different temperatures. The results are shown in Table IV. We have included the heats of ionization of the various ionizing groups of the reactants. These have been calculated from Table III and the equation given by Harned and Embree.

The values of ΔH for A , B , and C have little significance. They are reported merely to indicate that their order of magnitude is

TABLE III
Dissociation Constants and Temperature

Constants	θ	$pK_{\max.}$	25°	78°	100°	118°
pK_1	43	2.16	2.19	2.22	2.32	2.44
pK_2	25	4.32	4.32	4.46	4.60	4.76
$pK_B = pK_w - pK_2$	91	3.77	3.99	3.78	3.77	3.81
pK_w			13.93	12.61	12.24	11.99
pK_3	25	3.32	3.32	3.46	3.60	3.76
pK_3 (Phosphoric)	43	7.19	7.22		7.35	

The acetate and citrate buffers were assumed to suffer the same displacement with temperature as pK_1 .

that which would be expected for the heats of ionization involved in the reactions of System II. These heats vary so rapidly with temperature that the van't Hoff equation is scarcely applicable. It would appear, however, that the formation of the amide link by the uncharged condensing groups is a reaction involving very little energy change.

The critical increments may be examined from the point of view of absolute rates of reaction. In recent years some success has attended the attempt to calculate absolute rates of reaction in solution by means of the gas equation for collision frequency and the Arrhenius equation. If k is a velocity constant, $\log k = Ze^{-E/RT}$ where E is the activation energy and Z the collision number. We have given Z the plausible value 3×10^{11} and have

TABLE IV

Constant	Temperature range	pH ₃₅	ΔH			
Equilibrium constants						
	°C.					
$\frac{a_1}{a_2}$	78-100	0.98	3600			
	100-118	0.78	3200			
	100-118	1.73	3500			
$\frac{b_1}{b_2}$	78-100	2.4	500			
	100-118	2.4	1000			
	100-118	2.89	1000			
$\frac{c_1}{c_2}$	78-100	12.11	3800			
	100-118	11.87	4200			
	100-118	12.17	4200			
	100-118	12.73	3800			
Velocity constants						
			E'	E' corrected	E'_A	$E_{\text{calc.}}$
a_1	78-100	0.98	22,000	25,000	28,000	30,000
b_1	78-100	2.4	26,500	29,500	29,500	30,500
	100-118	2.89	26,500	29,000		
c_1	78-100	12.00	21,200	24,500	28,000	30,500
	78-100	13.13	21,800			
	78-100	9.99	21,500			
a_2	78-100	0.98	18,500		22,500	25,000
b_2	78-100	2.4	26,000		26,000	23,000
	100-118	2.89	26,000			
c_2	78-100	12.24	18,000		24,000	24,500
	78-100	13.13	18,500			
Heats of ionization (calculated from Table III)						
	K_1	K_2 and K_4	K_B			
78°	-2040	-3090	760			
100°	-3630	-4780	-570			
118°	-5250	-6500	-1890			

calculated the theoretical values of E corresponding to the observed velocity constants at 100°. These are compared in Table IV with the critical increment (E') calculated from the tempera-

ture coefficient of the velocity constant. In the cases of a_1 , b_1 , and c_1 a correction must be applied to E' to make it comparable with E . This is due to the fact that the reaction involves the molecules of the solvent and that the change in mean free path of the latter with temperature modifies the collision frequency. We have employed the equation given by Moelwyn-Hughes (11) which utilizes the change in viscosity for calculation of the correction. In Table IV " E' corrected" is the result. Only in the case of b_1 is there substantial agreement between the experimental critical increment and the theoretical activation energy. The value of b_2 is much greater than it would be were E' the activation energy. The other constants all define rates much slower than the number of activated collisions calculated from E' . Little significance can be attached to this, since there are a number of theoretical explanations possible for such discrepancies. We are more concerned with the fact that the critical increments of the three alternative reaction rates in either direction of System II differ from one another. Now, we have been able to relate the differences in reaction rates at a single temperature to the dissociation constants of the alternative reactants. It is tempting to seek a similar explanation for the differences between the critical increments. We have, accordingly, calculated the critical increments of a_1/K_1 , b_1 , and $K_2K_3c_1$ (cf. Equation 1) and, also, a_2/K_4 , b_2 , and $K_w c_2$ (cf. Equation 2) over the temperature range 78–100°. These have been designated E'_A and are shown in Table IV.

We see that Equations 1 and 2 do lead to a partial reconciliation of the various temperature coefficients. Moreover, the values of E'_A are now in more satisfactory agreement with the theoretical activation energies. The case of b_2 is the least satisfactory. We would remark that this is the one constant which it has been necessary to evaluate indirectly. Moreover, its values have been derived from data at a pH range in which the effects of a_2 and b_1 overlap somewhat. Little reliance can be placed on its temperature coefficient.

We have presented the above empirical analysis of critical increments merely to suggest that the temperature coefficients of the various hypothetical reactions tend to confirm the conclusion of the studies at a single temperature, that the kinetics

of the system are a simple function of the free energies of the various proton dissociations exhibited by the reactants. We hope to examine this important relationship by a more adequate study of the effects of temperature on the system.

In conclusion we would report, without detail, that we have compared System I-a with System II. It will be obvious from the discussion that System I-a (or I-b) must be as consistent with the relations of velocities and equilibria to pH at a single temperature as is System II. The alternative systems differ only in the reaction which they assume to be the rate-controlling process in the dehydration of glutamic acid. We have set up the equations for System I-a and derived the values at 78–100° of the velocity constants which are the counterparts of a_1 , b_1 , and c_1 in System II. The critical increments of these constants are all much greater than the activation energies calculated from the actual values at 100°. In other words, the observed rates of reaction are much greater than the number of activated collisions between the reactants of System I-a, assuming that E' is the activation energy. In System II the observed rates are comparable to or less than the collision frequency calculated from the corrected E' . To this extent the latter is the more plausible mechanism. Knowing nothing of the temperature coefficients of the various submerged ionizations involved in System I-b, we may not extend this method of analysis to System I-b.

SUMMARY

1. Observations are reported on the equilibrium and velocity constants in the reversible system glutamic acid-pyrrolidonecarboxylic acid in dilute aqueous solution. The results cover the whole pH range in which observations have been practicable and include three temperatures.

2. The mechanism of the reaction has been considered and it is suggested that the hypothesis that the reaction is a slow proton transfer is a useful one.

3. The dissociation constant of pyrrolidonecarboxylic acid has been measured and those of glutamic acid confirmed. The effects of ionic strength on these constants are reported.

4. Some observations on the use of the reaction in the routine of protein analysis are reported.

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STUDIES IN KETOGENESIS*

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The recent application of the tissue slice technique to the problem of ketogenesis by Quastel and Wheatley (1), Jowett and Quastel (2-4), and Edson (5, 6) has thrown considerable light on certain aspects of the mechanisms concerned in ketogenesis and antiketogenesis.

In this study twenty-two related compounds have been investigated in an attempt to elucidate further the mechanism of ketogenesis and antiketogenesis in the liver.

Methods

Rat liver slices from healthy, well fed male rats, 5 to 8 months of age, were used in a Barcroft-Warburg apparatus. The tissue slices, 10 to 20 mg. of dry weight, were immersed in a Ringer-phosphate buffer solution of pH 7.4 to which was added the substrate, previously neutralized to pH 7.4. The α -keto acids were synthesized according to the method of Mebus (7), and *dl*- β -hydroxybutyric acid according to Wisclicenus (8). The α -hydroxy acids were obtained from Dr. E. J. Witzemann.

Acetoacetic acid was determined with some modifications by the Van Slyke procedure as used by Edson (5). The method was found to be specific for acetone in the presence of such other ketones as might be formed as a result of the oxidation of the substrates studied, provided filtration of the mercury-acetone compound was carried out while *hot*. Certain of the substrates reacted with Denigès' reagent. Therefore, the prepared filtrates from control experiments were refluxed with the same amount

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of substrate as was originally added to the non-control experiment.

Procedure

Three manometers were used and so set up that for every three experiments (tissue slices plus substrate) two controls (equal weight of tissue slices without substrate) were available on the same rat liver. With this set-up, the acetoacetic acid production and the oxygen consumption of the liver slice in the presence of substrate, as well as the oxygen consumption of the liver slice in the presence of substrate *over* that of an equal weight of liver slice in the absence of substrate, were determined.

Thus in all cases the acetoacetic acid values reported represent an average of at least three experiments with an average of two controls on the same rat liver.

Units

- QO_2 = c.mm. of oxygen consumed per mg. of tissue (dry weight) per hour
 ΔQO_2 = c.mm. of oxygen consumed per mg. of tissue (dry weight) per hour in the experimental flask (substrate present) over an equal weight of tissue in the control flask (no substrate present)
 $QAcOAc$ = c.mm. of CO_2 equivalent to acetoacetic acid formed per mg. of tissue (dry weight) per hour
 $QKet$ = c.mm. of CO_2 equivalent to total ketones (acetoacetic acid plus β -hydroxybutyric acid) formed per mg. of tissue (dry weight) per hour

The subscript *R* refers to control flask. The subscript *L* refers to experimental flask.

Results

Oxidation of Normal Saturated Fatty Acids—Of this group (Table I) butyric and caproic acids are most actively ketogenic, acetic acid is slightly ketogenic, while propionic acid is antiketogenic. Valeric acid is neither ketogenic nor antiketogenic.

Oxidation of Aliphatic Amino Acids—Of the amino acids studied (Table II), *dl*- α -aminovaleric acid is the most strongly ketogenic, and *dl*-leucine slightly less so. The other amino acids are antiketogenic, with the exception of α -aminoisobutyric acid, which is neither ketogenic nor antiketogenic.

Edson (6) has reported leucine, norleucine, valine, and α -aminobutyric acid to be ketogenic with rat liver slices from normal animals. With the exception of leucine, the above are not in agreement with the results obtained here. Embden and Marx (9) showed by liver perfusion that α -aminovaleric acid was ketogenic, while α -aminobutyric and α -aminocaproic acids were anti-

TABLE I
Oxidation of Normal Saturated Fatty Acids

Acid, 0.01 M	QO ₂	Δ QO ₂	QAcoAco _R	QAcoAco _L
CH ₃ COOH.....	-12.08	-2.89	0.61	0.89
CH ₃ CH ₂ COOH.....	-11.73	-2.13	0.57	0.40
CH ₃ CH ₂ CH ₂ COOH.....	-12.18	-4.33	0.47	4.56
CH ₃ CH ₂ CH ₂ CH ₂ COOH.....	-13.30	-4.80	0.38	0.42
CH ₃ CH ₂ CH ₂ CH ₂ CH ₂ COOH.....	-17.17	-9.70	0.87	4.36

TABLE II
Oxidation of Amino Acids

Liver	Amino acid (dl-)	Concen- tration M	QO ₂	Δ QO ₂	QAcoAco _R	QAcoAco _L	QKet _R	QKet _L
Rat	CH ₃ CH ₂ CH(NH ₂)COOH	0.01	-9.65	-2.54	0.80	0.24	1.24	0.51
	"	0.02	-11.75	-2.02	0.41	0.26		
	(CH ₃) ₂ C(NH ₂)COOH	0.02	-3.84	0	0.68	0.68		
	CH ₃ CH ₂ CH ₂ CH(NH ₂)- COOH	0.02	-10.75	-3.82	0.78	2.87		
	(CH ₃) ₂ CHCH(NH ₂)- COOH	0.02	-9.56	-1.42	0.63	0.32		
	CH ₃ CH ₂ CH ₂ CH ₂ CH- (NH ₂)COOH	0.02		-3.46	1.10	0.63		
	(CH ₃) ₂ CHCH ₂ CH(NH ₂)- COOH	0.02	-10.75	-1.83	0.39	1.76		
Guinea pig	CH ₃ CH ₂ CH(NH ₂)COOH	0.02	-4.81	-0.94	0.25	0.16		

ketogenic. The antiketogenic action of α -aminobutyric acid is further shown (Table II) in guinea pig liver. Since only acetoacetic acid was determined, it was necessary to make sure that the apparent antiketogenesis was not the result of an increase in β -hydroxybutyric acid at the expense of acetoacetic acid. Therefore total ketones were determined in one experiment

(Table II). As is seen, the Q_{Ket_R} value was more than twice the Q_{Ket_L} value, thus demonstrating a true antiketogenesis.

α -Aminoisobutyric acid is apparently not metabolized and from the low QO_2 value appears to be toxic. Keilin and Hartree (10) have shown that α -aminoisobutyric acid is not deaminized by *d*-aminodeaminase.

TABLE III
Oxidation of α -Hydroxy and α -Keto Acids

Acid	Concentration	QO_2	ΔQO_2	Q_{AcOAc_R}	Q_{AcOAc_L}
	<i>M</i>				
$CH_3CH_2CHOHCOOH$	0 01	-8.42	-2.51	0.54	0.14
$CH_3CH_2COCOCH_3$	0 01	-9.61	-2.82	0.79	0.18
$(CH_3)_2C(OH)COOH$	0 01	-4.85	0	1.04	1.20
$CH_3CH_2CH_2CHOHCOOH$	0 01	-13.70	-1.64	0.95	1.72
$CH_3CH_2CH_2COCOCH_3$	0.005	-14.25	-3.43	0.52	2.00
$CH_3CH_2CH_2CH_2CHOHCOOH$	0 01	-9.45	-3.39	0.88	0.91

TABLE IV
Relationship of 4-Carbon Acids

Acid	Concentration	QO_2	ΔQO_2	Q_{AcOAc_R}	Q_{AcOAc_L}
	<i>M</i>				
$CH_3CH_2CH_2COOH$	0.01	-12.18	-4.33	0.47	4.56
$CH_3CH=CHCOOH$	0.01	-11.26	-3.35	1.29	6.47
$CH_3CHOHCH_2COOH$	0.01	-11.07	-1.09	1.31	6.11
$CH_3CH_2CH(NH_2)COOH$...	0.02	-11.75	-2.02	0.41	0.26
$CH_3CH_2CHOHCOOH$	0.01	-8.42	-2.51	0.54	0.14
$CH_3CH_2COCOCH_3$	0 01	-9.61	-2.82	0.79	0.18
$(CH_3)_2C(NH_2)COOH$	0.02	-3.84	0	0.68	0.68
$(CH_3)_2C(OH)COOH$	0.01	-4.85	0	1.04	1.20

The relationship of the branched α -amino acids from 4 to 6 carbon atoms is seen in the case of α -aminoisobutyric, α -aminoisovaleric, and α -aminoisocaproic acids. The first of these is not metabolized, the second is antiketogenic, and the third is ketogenic.

Oxidation of α -Keto and α -Hydroxy Acids— α -Keto- and α -hydroxyvaleric acids are the only ketogenic compounds of this group (Table III). α -Keto- and α -hydroxybutyric acids are anti-

ketogenic. At the concentration studied, α -hydroxycaproic acid is neither ketogenic nor antiketogenic. α -Hydroxyisobutyric acid is apparently not at all metabolized, and from its low QO_2 value appears to be toxic.

It is to be noted that α -ketovaleric acid at 0.005 M concentration is more strongly ketogenic than hydroxyvaleric acid at 0.01 M concentration. α -Ketovaleric acid at 0.01 M concentration was found to be toxic. It would also appear that α -ketobutyric acid is more strongly antiketogenic than an equal concentration of α -hydroxybutyric acid.

It is apparent here that the α -keto and α -hydroxy derivatives of the same fatty acid act similarly as far as ketogenesis and anti-ketogenesis are concerned.

Relationship of the 4-Carbon Acids— α substitution by an amino, hydroxy, or keto (Table IV) group makes the strongly ketogenic butyric acid antiketogenic.

It is to be noted that α -ketobutyric acid is more strongly antiketogenic than α -amino- and α -hydroxybutyric acid. This is in keeping with the concept that the α -keto acids are intermediates in the metabolism of α -amino and α -hydroxy acids.

Crotonic acid has a higher $QAcOAc$ value than β -hydroxybutyric and butyric acids. This may be explained by assuming that crotonic acid is an intermediate for these two acids in their oxidation to acetoacetic acid, as has been suggested by Dakin (11), or that the two different oxidative pathways known to exist for butyric and β -hydroxybutyric acids are both simultaneously available to crotonic acid. The second of these two possibilities has some experimental evidence in its favor. Wakeman and Dakin (12) showed that liver brei would oxidize β -hydroxybutyric acid to acetoacetic acid, but would not oxidize butyric acid. Jowett and Quastel (2) by means of inhibitors have shown that crotonic acid acted as though it were oxidized by the same enzyme system as butyric acid. On the other hand, Friedmann and Maase (13) have shown that liver brei would convert crotonic acid to β -hydroxybutyric acid. This must mean that crotonic acid is available to both these oxidative enzyme systems. This fact would then account for the high $QAcOAc$ values of crotonic acid as compared with butyric and β -hydroxybutyric acids. This relationship is being further investigated at present.

Relationship of the 5-Carbon Acids—The effect of substitution by an amino, hydroxy, or keto group on valeric acid is seen from Table V. α substitution by any of the above groups results in a ketogenic compound. The QAcOAc of α -ketovaleric acid at 0.005 M concentration is somewhat less than the QAcOAc of α -aminovaleric acid at 0.02 M, showing that α -ketovaleric acid is more strongly ketogenic than α -aminovaleric acid. Here again it is apparent that the α -keto acid of the series acts as the metabolic intermediate for the α -hydroxy and α -amino acid.

The rôle of branched chain compounds is to be seen in the case of isovaleric acid and *dl*-valine. Thus isovaleric acid is ketogenic, while α -aminoisovaleric acid (*dl*-valine) is antiketogenic. This

TABLE V
Relationship of 5-Carbon Acids

Acid	Concen- tration	QO ₂	Δ QO ₂	QAcOAc _R	QAcOAc _L
	M				
CH ₃ CH ₂ CH ₂ CH ₂ COOH.....	0.01	-13.30	-4.80	0.38	0.42
CH ₃ CH ₂ CH ₂ CH(NH ₂)COOH.....	0.02	-10.75	-3.82	0.78	2.87
CH ₃ CH ₂ CH ₂ CHOHCOOH.....	0.01	-13.70	-1.64	0.95	1.72
CH ₃ CH ₂ CH ₂ COCOOH.....	0.005	-14.25	-3.43	0.52	2.00
CH ₃ COCH ₂ CH ₂ COOH.....	0.01	-4.47	0	5.72	7.03
(CH ₃) ₂ CHCH ₂ COOH.....	0.01	-10.35	-3.61	0.78	2.88
(CH ₃) ₂ CHCH(NH ₂)COOH.....	0.02	-9.56	-1.42	0.63	0.32

demonstrates the ability of the liver to demethylate. In the case of isovaleric acid, butyric acid, which is ketogenic, is probably an intermediate, while with *dl*-valine, α -aminobutyric acid, which is antiketogenic, is probably an intermediate. However, whether demethylation precedes oxidation, or follows it, is not apparent from these data. These results on branched chain compounds are in agreement with the early studies of Baer and Blum (14) and Friedmann (15).

Levulinic acid is toxic, as is seen from the low QO₂ value. The difference in QAcOAc values between the control and experimental data is not considered significant, since the data overlap in individual determinations. It is apparent that this compound readily reacts with Denigès' reagent.

Relationship of the 6-Carbon Acids—From Table VI it is seen that α substitution by an amino group in caproic acid makes this ketogenic compound antiketogenic. This effect is similar to that with butyric acid. The fact that α -hydroxycaproic acid does not act in a similar manner would suggest that its dehydrogenation to α -ketocaproic acid is much slower than the deamination of α -aminocaproic acid. Bernheim (16) has shown that dehydrogenation of α -hydroxy acids by lactic acid dehydrogenase was much slower for α -hydroxybutyric acid than for lactic acid.

TABLE VI
Relationship of 6-Carbon Acids

Acid	Concen- tration	QO_2	ΔQO_2	$QAcoAcE$	$QAcoAcL$
	<i>M</i>				
$CH_3CH_2CH_2CH_2CH_2COOH$	0.01	-17.17	-9.70	0.87	4.36
$CH_3CH_2CH_2CH_2CH(NH_2)COOH$	0.02		-3.46	1.10	0.63
$CH_3CH_2CH_2CH_2CHOHCOOH$	0.01	-9.45	-3.39	0.88	0.91
$CH_3CH=CHCH=CHCOOH$	0.01	-9.14	-3.33	2.43	6.03
$(CH_3)_2CHCH_2CH(NH_2)COOH$	0.02	-10.75	-1.83	0.39	1.76

TABLE VII
Antiketogenic Action of α -Aminobutyric Acid

α -Aminobutyric acid	Butyric acid	ΔQO_2	$QAcoAcE$	$QAcoAcL$
<i>M</i>	<i>M</i>			
0.01	0.01	-0.96		1.84
	0.01		3.76	

While the higher homologues were not studied, it might be expected that these would be dehydrogenated at a progressively slower rate.

Sorbic acid, the 6-carbon homologue of crotonic acid, is seen to be strongly ketogenic, and to have a $QAcoAc$ value of the same order as caproic acid. Whether or not sorbic acid can be converted to β,Δ -dihydroxycaproic acid by liver brei, in a manner analogous to the conversion of crotonic acid to β -hydroxybutyric acid, has not been determined.

Again, the rôle of branched chain compounds is seen in the case of leucine. After deamination and demethylation, α -ketovaleric acid, which is ketogenic, would presumably result.

Antiketogenic Action of α -Aminobutyric Acid in Presence of Butyric Acid—When equal concentrations of α -aminobutyric acid and butyric acid are simultaneously metabolized (Table VII), the QAcOAc value of butyric acid is lowered by one-half, or in other words, antiketogenesis occurs.

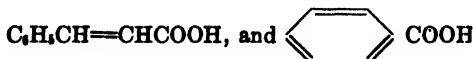
DISCUSSION

It is well established that a special enzyme system independent of the intact liver cell exists for the oxidation of β -hydroxybutyric acid to acetoacetic acid, and for the reduction of acetoacetic acid to β -hydroxybutyric acid. Whether or not this represents a single reversible system, or two distinct systems, is not known with certainty. However, very little is known regarding the enzyme systems responsible for the oxidation of fatty, amino, and other substituted fatty acids. It is well established to date that the oxidation of butyric acid requires the intact liver cell. Thus, it has not been possible to study the oxidation of such compounds by extracts, breis, etc. However, from the studies of intact biological systems, and more recently of tissue slices, certain facts tend more or less to characterize such an enzyme system.

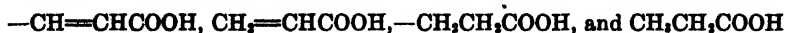
The fact that β oxidation in the sense of Knoop has come into more or less acceptance has suggested all along that a selective oxidation exists which is characterized in the intact cell by an oxidation initiated β to the carboxyl group. The apparent exceptions to the β oxidation scheme have led to many other schemes for an explanation. While it is well known that ketogenesis is not the result of the oxidative breakdown of higher fatty and substituted fatty acids alone, and that many factors are involved which will alter such an oxidative course, yet it would seem that some characteristic oxidative pathway must exist for related chemical compounds. If a specific enzyme system is concerned in oxidative ketogenesis, then the ketogenic compounds must have certain definite chemical groupings in common. On the other hand, if certain compounds are antiketogenic, or inhibit ketogenesis more or less specifically by such an oxidative pathway, then these compounds, too, must have a specific chem-

ical grouping in common with the ketogenic compounds, for obviously the same enzyme surfaces must be available to both.

The recent inhibition studies of Jowett and Quastel (3) have shown the following compounds to inhibit the ketogenesis of a known ketogenic compound.



If these compounds are examined it will be seen that the following related groups are in common.



While benzoic acid at first glance appears to be an exception, actually a $-\text{CH}=\text{C}-\text{COOH}$ group is present. Of these com-

pounds benzoic acid alone is not oxidized, and this, possibly, is due to the fact that no α -hydrogen atom is present. Jowett and Quastel found benzoic, phenylpropionic, and cinnamic acids to be the strongest inhibitors, the latter two compounds producing benzoic acid on oxidation. The toxicity of benzoic acid may well be postulated on such a basis. That is, the enzyme surface cannot give up benzoic acid by oxidation and therefore conjugation with glycine or glucuronic acid is necessary.

The reason that propionic acid is antiketogenic in the presence of ketogenic compounds is obvious. Propionic acid is using the same enzyme system as the compound which is ketogenic. As a result we have a case of competition between two substrates, one of which can form acetoacetic acid, while the other cannot, with a resulting antiketogenesis. Quastel and Wheatley (1) have shown that as the propionic acid concentration in the presence of butyric acid is increased, the QAcOAc from butyric acid is decreased.

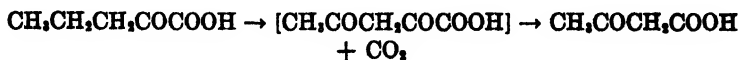
If the compounds $\text{CH}_3\text{CH}_2\text{COCOOH}$ and $\text{CH}_3\text{CH}_2\text{CH}_2\text{COCOOH}$ are considered as being the metabolic intermediates for the corresponding hydroxy and amino acids, it is seen that the presence of the carbonyl group makes the grouping $-\text{CH}_2\text{CH}_2\text{COOH}$ impossible. And yet one of these compounds is ketogenic and the other antiketogenic, as can be seen from Table III. Embden

and his coworkers, who early studied the metabolism of amino acids by the perfusion method, believed that after deamination the resulting α -keto acids were decarboxylated, leaving an aldehyde which on oxidation would yield the corresponding acids of 1 carbon atom less. According to this, α -ketovaleric acid would yield butyric acid which is ketogenic, while α -ketobutyric acid would yield propionic acid which is antiketogenic. However, the fact that animal tissues show very little carboxylase activity would make the aldehyde intermediate unlikely. More probably oxidation and decarboxylation occur simultaneously ("oxidative decarboxylation") (17).

The fact that in the liver α -keto acids are reduced to α -hydroxy acids and are reaminated to amino acids is sufficient evidence for the availability of the intact α -keto acid molecule for the various enzyme surfaces.

In attempting to find a chemical grouping common to both the inhibitory, or antiketogenic, and ketogenic substances, it will be seen that the following grouping is present in all these: $\text{CH}=\text{CHCO}$, or $\text{CH}_2\text{CH}_2\text{CO}$. In other words, if one considers a carbonyl group to be required rather than a carboxyl, one can then explain the ketogenesis and antiketogenesis of the α -keto acids.

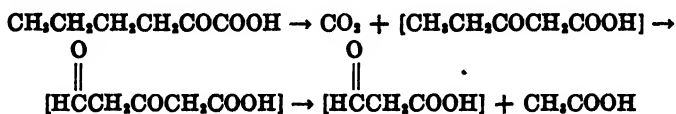
If the enzyme system, here called β -oxidase, requires the presence of the chemical grouping $\text{CH}=\text{CHCO}$, or $\text{CH}_2\text{CH}_2\text{CO}$, the explanation for the ketogenesis of α -ketovaleric acid and the antiketogenesis of α -ketobutyric acid is easily understood. This chemical grouping required by the β -oxidase system is present in both. The carbonyl group may be considered as the polar or orienting group in the molecule. If 2 carbon atoms in a row, having at least 1 hydrogen atom on each carbon atom, are adjacent to the carbonyl group, oxidation will occur β to this carbonyl group. Thus in the case of both α -ketobutyric and α -ketovaleric acids, oxidation will occur β to the carbonyl group, but γ to the carboxyl group. Oxidative decarboxylation will take place simultaneously according to the following.



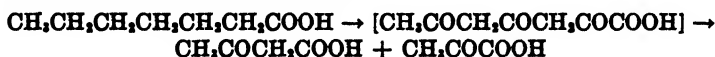
While the nature of the end-products of the oxidation of a compound like α -ketobutyric acid is not known, the following analogous course may be postulated.



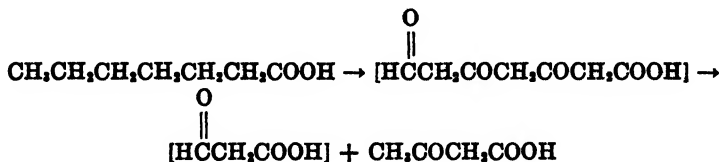
The nature of these end-products will be discussed further. The oxidation of α -ketocaproic acid could be considered as taking place as follows:



This idea of successive β oxidation is in line with the evidence available for the oxidative breakdown of fatty and aliphatic amino acids. Jowett and Quastel (3) include in their scheme of alternate oxidation the possibility of α , γ oxidation to explain the slight ketogenesis of the higher, odd numbered fatty acids. Thus they would postulate for heptylic acid:



Yet a similar result could be obtained by successive β oxidation by the β -oxidase system.



And it is on this basis that the production of 2 moles of acetoacetic acid from octoic acid is explained by these authors.



Thus the carbonyl group may be considered the polar group in the molecule, in that oxidation will take place by the β -oxidase system with reference to this group. That is, oxidation will occur β to the carbonyl group.

The glucogenesis of the odd numbered fatty acids was demonstrated by Ringer (18). The higher homologues of propionic acid were further shown to be glucogenic only with respect to their ability to yield propionic acid or its equivalent. More recently Deuel, Butts, Hallman, and Cutler (19) have shown that the odd numbered homologues of propionic acid are glycolytic when fed to rats. These odd numbered fatty acids must be oxidized by the same enzyme system (β -oxidase) as the even numbered fatty acids, since they inhibit the ketogenesis of the latter in the presence of each other, as shown by Quastel and Wheatley (1) and Edson (20).

The antiketogenic and glucogenic action of propionic acid can be explained as a result of oxidation by the β -oxidase system according to the following scheme.



By the addition of water to β -hydroxyacrylic acid, glyceric acid will be formed.



The evidence for this scheme is as yet not available, but is at present being investigated. The slight ketogenesis of heptylic and the other odd numbered fatty acid in the absence of strongly ketogenic compounds, the relative antiketogenic action in the presence of a strongly ketogenic compound, and the ability to yield glucose upon oxidation to the extent of 3 carbon atoms are all explainable by the oxidation scheme proposed (see previous scheme for heptylic acid oxidation).

Edson's (20) recent studies on substrate competition in rat liver slices has clearly indicated that many different substances can indirectly affect the ketogenesis from a given ketogenic compound. Edson suggested that these different compounds are competing with fatty acids for available oxygen. This serves to emphasize the many different oxidative mechanisms which may influence a given oxidative pathway. However, this does not invalidate the concept that a given oxidative system is limited to the oxidation of certain related chemical compounds. Until

the β -oxidase system suggested here is isolated, absolute proof for its existence is obviously not possible. However, it would seem from the evidence available to date that such a system exists.

SUMMARY

1. In considering the chemical nature of the compounds studied in relation to their ketogenesis or antiketogenesis, it is felt that a definite chemical grouping is required for oxidation by an oxidizing enzyme system, here called the β -oxidase system. This particular grouping is present also in those compounds which Jowett and Quastel found to inhibit more or less specifically the oxidation of known ketogenic compounds. The specific group involved is reported here, in skeleton form, to be as follows: $-\text{CH}=\text{CHC}=\text{O}$. Thus, the group requires 3 carbon atoms with at least 1 hydrogen atom on each carbon atom. The adjacent carbon atoms may have their full complement of hydrogen atoms, as in a $\text{CH}_3\text{CH}_2\text{CO}-$ or $-\text{CH}_2\text{CH}_2\text{CO}-$ group, and the carbonyl group may exist as such, or be present in a carboxyl group, thus $-\text{CH}_2\text{CH}_2\text{COOH}$. With the carbonyl group acting as a polar or orienting group, oxidation will occur β to this carbonyl group.

2. A scheme of successive β oxidation, similar to that proposed by Jowett and Quastel, is offered to explain the oxidation of fatty, amino, and other substituted fatty acids. Each newly formed carbonyl group acts as a polar group for the β -oxidase system.

3. A scheme is suggested to explain the antiketogenesis, slight ketogenesis, and glucogenesis of the higher odd numbered fatty acids on the basis of oxidation by the β -oxidase system. This scheme involves the hypothetical intermediates of β -hydroxy-acrylic acid and glyceric acid formed as a result of β oxidation of the terminal 3 carbon atoms of the odd numbered fatty acid chain.

The author wishes to express his indebtedness to Miss Katherine Baird and Miss Irene Stark, who gave capable technical assistance in certain parts of this study.

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DETERMINATION OF FERMENTABLE BLOOD SUGAR BY GASOMETRIC MEASUREMENT OF THE CARBON DIOXIDE FORMED BY THE ACTION OF YEAST

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Buchner and Mitscherlich (1904) demonstrated the constancy of the CO_2 produced from glucose by specific yeast preparations under given conditions. Grafe and Sorgenfrei (1924) and Lund and Wolfe (1926) used the Barcroft apparatus, and Van Slyke and Hawkins (1929) the Van Slyke-Neill apparatus, to determine the CO_2 produced by the action of yeast on blood and urine, as a measure of the fermentable sugar.

It has been the object of the present work to utilize this principle for development of a gasometric fermentation method, sufficiently rapid, accurate, and sensitive to be used in routine microanalyses.

The technique used is similar in principle to that in the gasometric urease urea method of Van Slyke (1927). CO_2 is produced from the substrate by enzymatic action in the manometric apparatus of Van Slyke and Neill. Quantitative CO_2 evolution is made almost instantaneous by rapid shaking and the use of a high ferment to substrate ratio, and the CO_2 is measured as in blood gas analyses. In the present method, suspensions of commercial pressed yeast are used as enzyme. The fermentation is carried out in dilute citric acid at an acidity which frees the evolved CO_2 , so that fermentation and extraction of the CO_2 in the evacuated manometric chamber can be performed simultaneously. The fermentation-extraction process is completed in 3 minutes, and a complete analysis can be run in 6 minutes. For the analyses, either blood filtrates or diluted whole blood may be used, with identical results, although the filtrates are more convenient to manipulate.

The earlier microreduction methods for blood glucose were subject to errors from the presence, in the blood filtrates analyzed, of non-fermentable reducing materials which caused reductions equivalent to 20 to 40 mg. per cent of blood glucose (Hiller, Linder, and Van Slyke, 1925; Somogyi, 1927). The error caused by these materials has been diminished by the introduction of sugar reagents, such as those of Benedict (1925, 1928) and Folin (1928, 1929), which are less affected by non-sugar substances in blood filtrates, and by the devising of protein precipitants which remove with the blood proteins a large part of the non-sugar reducing crystalloids (West, Scharles, and Peterson, 1929; Somogyi, 1930; Steiner, Urban, and West 1932). In normal bloods these devices have lowered the sugar values obtained by reduction methods to practically the level of the fermentable sugar. In uremic blood, however, even the latest reduction methods give results somewhat higher than the fermentable sugar (Miller and Van Slyke, 1936).

For estimation of the fermentable sugar Hiller, Linder, and Van Slyke (1925) introduced the procedure of determining the reducing material before and after fermentation with enough yeast to remove the glucose in 30 minutes, before bacterial action could affect the results. The difference indicated the fermentable sugar removed by the yeast. Somogyi (1927) improved the procedure by using washed yeast and carrying out the fermentation in blood filtrate instead of in whole blood. This procedure has become the standard of reference for determination of the "true sugar." It yields consistent results, but suffers the disadvantage of being a determination by difference, which makes the errors additive for the two analyses involved. The procedure is also relatively time-consuming, since it involves analyses of two samples, one of which must be first treated with, then separated from, the yeast, before analysis. The direct fermentation method proposed in the present paper yields, within the limits of analytical error, the same results as the combined fermentation-reduction procedure, and is simpler and quicker.

Two procedures will be described; a macroanalysis, which is carried out with 5 cc. of a 1:10 blood filtrate; and a microanalysis, which requires only 0.12 cc. of whole blood.

Reagents for Macro and Micro Blood Sugar Determinations

Suspension of Brewers' Yeast—Brewers' yeast is employed if it can be conveniently obtained. Its action is slightly quicker than that of bakers' yeast, and the CO₂ yield is higher. Pure strain brewers' yeast¹ is employed. To prepare the suspension,

¹ Fresh pressed brewers' type yeast No. 2019, supplied on special request by The Fleischmann Laboratories, 810 Grand Concourse, New York, was used in the experiments recorded in this paper. This strain of yeast, used in the preparation of vitamin B concentrates, is produced by

transfer 30 gm. of yeast to a 125 cc. Erlenmeyer flask, add 60 cc. of distilled water, stopper, and shake vigorously for 1 minute. The suspension is allowed to stand for 30 minutes before use, in order to permit attainment of a steady rate of endogenous CO_2 production,² to give a constant c correction (see below). It may then be used at any time for 6 hours.

Suspension of Bakers' Yeast—This is used if brewers' yeast is not obtainable. Commercial flour cake yeast³ is employed. To prepare the suspension, break up a cake of yeast and transfer it to a 125 cc. Erlenmeyer flask, add 20 cc. of distilled water, stopper, and shake vigorously for 1 minute. The suspension is allowed to stand for 30 minutes before use.

Citric Acid Reagent—21 gm. of citric acid and 10 gm. of NaCl are dissolved in distilled water and made up to 500 cc. The NaCl tends to suppress mold growth in the citric acid and does not affect the fermentation.

Antifoam Reagent—Equal volumes of phenyl ether and dioxane⁴ are mixed and kept in a dropping bottle provided with a capillary tip. These reagents are chemically inert, and are nearly as efficient as octyl alcohol, which cannot be used because it inhibits fermentation.

the Peekskill, New York, plant of Standard Brands, Inc., and is readily available only in the vicinity of New York City. It is supplied in 1 pound lots and keeps for 2 weeks at 5°. Suspensions kept in thermos bottles at 0.5° showed a slow rise in fermentive power, reaching a maximum in about 12 hours, when the yield of CO_2 from glucose was as high as 95 per cent of theoretical, compared with 86 per cent yielded by suspensions made up as directed and kept at room temperature.

² Hopkins and Roberts (1935) state that autofermentation is negligible in brewers' yeast 1 day old.

³ Fleischmann's yeast, distributed by Standard Brands, Inc. Cakes may be obtained at any grocery store. The weight of a single cake is quite uniformly about 13 gm., so that weighing of the yeast is not necessary. This yeast contains a small amount of added oily vitamin concentrate which floats to the surface of the suspension, but does not interfere with analyses.

⁴ These reagents were supplied by the Laboratories of the Eastman Kodak Company. The use of phenyl ether as a foam inhibitor is due to Mitchell and Eckstein (1918). The dioxane (1:4 dioxane) serves as an inert water-soluble diluent for the phenyl ether and appears to increase its foam-inhibiting power.

Sodium Hydroxide—5 N.

Methyl Orange—0.1 per cent in 50 per cent alcohol. (In a dropping bottle.)

Citric Acid—Saturated solution (approximately 6 M). (In a dropping bottle.)

Procedure

Macromethod

Precipitation of Blood—Conventional 1:10 filtrates prepared according to either Somogyi (1930) or Folin and Wu (1919) are satisfactory.

Removal of Preformed CO₂ from Filtrates—For duplicate analyses, about 15 cc. of filtrate are run into a 125 cc. Erlenmeyer flask. 1 drop of methyl orange indicator is added, then saturated solution of citric acid, drop by drop, until a permanent pink color results. Not more than 2 drops should be required. The filtrate is whirled vigorously in the flask for 30 or 40 seconds to facilitate the escape of CO₂. Air is drawn through the flask for a few seconds to sweep out the liberated CO₂, as described by Van Slyke ((1927) p. 704) for the manometric urease urea method. The rotation and aeration are repeated in the same manner twice more. Repeated experiments with 4 mM bicarbonate solution in place of filtrate have demonstrated that this treatment will consistently reduce the CO₂ content of the solution to a constant limiting value.⁵ Several flasks may be grasped in one hand and whirled at the same time. The filtrates are then ready for analysis.

Removal of Preformed CO₂ from Yeast—3 cc. of yeast suspension, shaken well beforehand, and 2 cc. of citric acid reagent are run into the cup of the manometric apparatus and admitted into the chamber, followed by 1 drop of antifoam reagent. The chamber is evacuated and shaken for 1½ minutes to remove the preformed CO₂. The extracted gas is then ejected from the chamber. Air is admitted into the chamber during the ejection in order to prevent reabsorption of CO₂, as described by Van Slyke ((1927) pp. 705, 706) for the manometric urease urea method.

⁵ This limiting value is a CO₂ content which yields a pressure of about 4 mm. of mercury at 2 cc. volume and 25°, when 5 cc. of the solution are analyzed as described below. It appears to represent a state of equilibrium of the acidified solution with atmospheric CO₂.

This procedure does not remove all of the CO_2 present, but is effective in reducing its content to a constant level for a given yeast suspension. It does not interfere with the ability of the yeast to ferment glucose during the time required for an analysis.

The yeast suspension and citric acid must be freshly mixed in the manometric chamber for each analysis. Also, *as soon as the preformed CO_2 has been removed from the yeast, the sugar solution must be added and the analysis carried through to a finish.* A delay at this point would cause error for two reasons: (1) the respiration of the yeast would replace some of its "preformed CO_2 ," which has just been removed; (2) the citric acid would in time injure the fermenting activity of the yeast.

Simultaneous Fermentation and CO_2 Extraction—The cup over the manometric chamber is rinsed and 5 cc. of the blood filtrate, freed of preformed CO_2 as previously described, are admitted at once into the chamber from a stop-cock pipette, as described by Peters and Van Slyke ((1932) p. 240, Fig. 30). The chamber is then evacuated and shaken for 3 minutes, if brewers' yeast is used. If bakers' yeast is used, the chamber is shaken 4 minutes at temperatures over 25° ; 5 minutes for macroanalyses below 25° . During this time fermentation is completed and the CO_2 produced is extracted. At the end of the 3 to 5 minute shaking, the gas volume in the chamber is brought to 2 cc., with the technique described by Peters and Van Slyke ((1932) p. 277) for adjustment of gas volume in CO_2 analyses. The reading p_1 is then taken.* Then 0.5 cc. of 5 N NaOH is admitted into the chamber under slight negative pressure, to absorb the extracted CO_2 , as in the determination of plasma CO_2 , describe by Peters and Van Slyke ((1932) p. 284). The meniscus is then lowered below the 2 cc. mark for 30 seconds to allow completion of drainage down the walls of the chamber. Finally the meniscus is returned to the 2 cc. mark and p_0 is read. The manometric chamber is washed once with water between analyses, according to Peters and Van Slyke ((1932) p. 236).

The periods of 3 and 4 minutes are the shortest in which maximum yields of CO_2 could be obtained with the brewers' and bakers'

* It is essential to have a good source of light behind the chamber when locating the meniscus, owing to the opacity of the yeast suspension. See "Addendum," p. 367.

yeast used. It is desirable that each analyst determine for the yeast he uses, by experiments like those of Table IV, the time that is necessary for maximal CO_2 yield. The fermentation-extraction period should be just long enough for this yield. Use of a longer period would unnecessarily increase the c correction, owing to CO_2 formation by yeast from reactions other than sugar fermentation.

Determination of Blood Sugars Exceeding 300 Mg. Per Cent—As shown later (Table VI) the CO_2 yielded per mg. of glucose in 3 minutes decreases somewhat if the amount of glucose present exceeds that in filtrates from blood containing 300 mg. per cent. Consequently, for such hyperglycemic blood, the factors in Table I may be a few per cent too low. When such a high sugar content is encountered, if strictly accurate results are desired a second portion of filtrate is diluted with 1 or 2 volumes of water. The analysis is then repeated on the diluted filtrate and the dilution factor is included in the calculation.

Blank Analysis—To determine the c correction (Peters and Van Slyke (1932) p. 279) a blank analysis is run in which 0.5 per cent Na_2SO_4 solution replaces the blood filtrate. The c correction represents chiefly CO_2 formed in the yeast, by processes other than fermentation of sugar, during the part of the analysis between "removal of preformed CO_2 from yeast" and completion of the 3 minute "simultaneous fermentation and CO_2 extraction." The value obtained in the blank analysis for $p_1 - p_0$ is taken as the c correction.

The c correction is constant for a given fresh suspension of brewers' yeast for at least 6 hours.⁷ Its value varies slightly from one suspension to another, presumably because of variations in the number of live yeast cells per unit volume. With the strain of brewers' yeast employed in this work, its value at 25° was about 30 mm. in the macromethod and 40 mm. in the micro-method. Experiments with other strains of yeast indicated that the c correction is fairly characteristic, although not as constant as the fermentive ability of a given strain under the conditions described. It varies considerably with temperature. For this reason, during a given series of analyses, the temperature must

⁷ Stier and Stannard (1936), working with bakers' yeast suspended in non-nutrient media, have demonstrated an initial period of constant rate of CO_2 production lasting 4 to 5 hours.

be held constant at that obtaining when the c correction is determined, within limits of $\pm 0.5^\circ$. Adjustment of the manometric chamber temperature is easily accomplished by applying hot or cold wet towels to the water jacket. The temperature of the chamber could be kept constant by passing through the water jacket a stream of water from a thermoregulated tank, but we have not found this necessary.

Since the c correction is a function of time, it is important that the *time interval*, from the end of "removal of preformed CO_2 from yeast" to the end of "simultaneous fermentation and CO_2 extraction," should be the same in each analysis and in the blank. Regularity in timing becomes automatic after a little practise.

Calculation of Results— P_{CO_2} , the pressure, measured at 2 cc. volume (or, for microanalyses, at 0.5 cc. volume) of CO_2 formed from glucose in the sample, is calculated as

$$P_{\text{CO}_2} = p_1 - p_0 - c$$

Whence the fermentable sugar (estimated as glucose) is calculated as

$$\text{Mg. glucose per 100 cc. blood} = P_{\text{CO}_2} \times \text{factor}$$

The factor is obtained from Table I.

Example—An analysis of normal blood by the macromethod with brewers' yeast gave the following figures: $p_1 = 107.8$ mm.; $p_0 = 45.8$ mm.; $c = 29.4$ mm. Temperature = 24.0° . From Table I the factor for 24.0° is 2.787. Hence

$$\begin{aligned} \text{Mg. glucose per 100 cc. blood} &= (107.8 - 45.8 - 29.4) \times 2.787 \\ &= 32.6 \times 2.787 = 91 \end{aligned}$$

To obtain factors for calculating the mg. of glucose in the sample analyzed, divide the factors in Table I for the macromethod by 200, and those for the micromethod by 1300.

The factors for use with brewers' yeast in Table I were calculated as follows: Van Slyke and Sendroy (1927) have given the calculation of F for the formula

$$\text{mm CO}_2 = P_{\text{CO}_2} \times F$$

where mm CO_2 indicates the mm of CO_2 in a sample of solution extracted in the Van Slyke-Neill chamber and F is a factor dependent on the temperature and the volume, S , of solution extracted. As shown in the experimental part of this paper, 1 mm, or 180 mg., of glucose, yields on the average 1.725 mm of CO_2 under the conditions of the macrodetermination, and 1.63

mm under the conditions of the microdetermination. Hence, for the macrodetermination,

$$\text{mm glucose} = \frac{F}{1.725} \times P_{\text{CO}_2}$$

$$\text{and mg. glucose} = \frac{180}{1.725} \times F \times P_{\text{CO}_2} = 104.3 \times F \times P_{\text{CO}_2}$$

The filtrate sample used in the macroanalysis represents 0.5 cc. of blood. Hence, to calculate mg. of glucose per 100 cc. of blood, the formula is,

$$\text{Mg. glucose per 100 cc. blood} = \frac{100 \times 104.3}{0.5} \times F \times P_{\text{CO}_2} = 20,860 \times F \times P_{\text{CO}_2}$$

Similarly, for the microdetermination, one calculates

$$\text{Mg. glucose per 100 cc. blood} = \frac{180}{1.63} \times \frac{100}{0.0769} \times F \times P_{\text{CO}_2} = 14,360 \times F \times P_{\text{CO}_2}$$

The factors in Table I are accordingly values of $20,860 \times F$ and $14,360 \times F$. The F values were calculated by the formula of Van Slyke and Sendroy (1927), with $S = 10$ and 3.5, and $a = 2.0$ and 0.5, for the macro- and micromethods, respectively.

The same procedure was used in calculating the factors for use with bakers' yeast, except that for it the average yields assumed per mole of glucose were 1.323 and 1.35 moles of CO_2 in the macro- and micromethods, respectively.

Application to Urine

For application to urine the above method requires no modification, except in preparation of the sample by dilution. The urine is so diluted that 5 cc. of the diluted material contain not more than 1.5 mg. of fermentable sugar. The solution thus prepared is analyzed as directed above for blood filtrate. For the calculation, the factor from Table I is multiplied by $\frac{\text{Dilution}}{10}$, in order to give mg. of sugar per 100 cc. of urine.

Micromethod

Precipitation of Blood—The dilute tungstic acid solution used by Van Slyke and Hawkins (1928) in the gasometric micromethod for reducing sugar in blood is recommended. 1 volume of 10 per cent sodium tungstate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$) is mixed with 8 volumes of $N/12$ sulfuric acid (*i.e.* mixed reagents of Folin and Wu (1919)). 2 volumes of this mixture are diluted with 3 volumes

of distilled water. The resulting dilute tungstic acid solution can be used for 2 weeks.

For *duplicate analyses*, 5 cc. of the dilute tungstic acid solution are measured into a centrifuge tube of about 12 cc. capacity. 0.2 cc. of blood is run into the tube from a capillary pipette calibrated "to contain," and the pipette is rinsed twice by drawing the tungstic acid up into it. For a *single analysis*, 0.12 cc. of blood is delivered similarly into 3 cc. of tungstic acid. The dilution factor is the same for both analyses. Any micro blood pipette of 0.1 or 0.2 cc. capacity can be calibrated at 0.12 cc. by weighing 1.62 gm. of mercury in the dry pipette. (To correct for approximately 0.5 mm. meniscus difference between mercury and water in a 1 mm. capillary, 1.62 instead of 1.627 gm. of mercury are taken (see Peters and Van Slyke (1932) Fig. 4, p. 20).) The centrifuge tube is stoppered and shaken to complete the blood protein precipitation, then centrifuged at 2500 R.P.M. for 10 minutes.

Removal of Preformed CO₂ from Blood Filtrate—After centrifuging, the clear supernatant fluid is decanted into a 30 cc. Erlenmeyer flask. If a few floccules of precipitate accompany the solution, they do no harm. A small drop of methyl orange indicator is added, then 1 drop of saturated citric acid solution. This should produce a permanent pink color; 2 drops may rarely be necessary. The flask is then whirled and aerated to sweep out CO₂, as in the macromethod. If the flask is then stoppered to prevent evaporation, the contents may be kept for several hours before analysis.

Removal of Preformed CO₂ from Yeast—1 cc. of yeast suspension and 0.5 cc. of citric acid reagent are shaken in the evacuated manometric chamber for 1½ minutes, and the extracted gas is ejected as described for the macromethod. No antifoam reagent is necessary.

Simultaneous Fermentation and CO₂ Extraction—2 cc. of the aerated blood filtrate are admitted from a stop-cock pipette into the chamber with the yeast, as in the macromethod. The filtrate sample should preferably contain not over 0.3 mg. of glucose, equivalent to 400 mg. per cent of blood sugar. Immediately after admission of the sample the chamber is evacuated and shaken, for 3 minutes with brewers' yeast, or for 4 minutes with

bakers' yeast. The reading of p_1 is then taken as described for the macromethod, except that here the reading is taken with the gas in the chamber at 0.5 cc. volume. 2 drops of 5 N NaOH are admitted to absorb the CO_2 , and p_0 is read with the residual gas at 0.5 cc. volume. Before the p_0 reading, the meniscus is kept for 30 seconds below the 0.5 cc. mark, to permit completion of drainage.

TABLE I

Factors by Which P_{CO_2} in Mm. Is Multiplied to Calculate Mg. of Fermentable Sugar in 100 Cc. of Blood

Temperature of gas chamber	Factors for brewers' yeast		Factors for bakers' yeast	
	Macromethod	Micromethod	Macromethod	Micromethod
	Sample = 0.5 cc. blood $S = 10$ cc. $a = 2$ cc.	Sample = 0.0769 cc. blood $S = 3.5$ cc. $a = 0.5$ cc.	Sample = 0.5 cc. blood $S = 10$ cc. $a = 2$ cc.	Sample = 0.0769 cc. blood $S = 3.5$ cc. $a = 0.5$ cc.
°C.				
20	2.880	4.376	3.755	5.283
21	56	53	24	56
22	32	29	3.692	27
23	08	09	61	03
24	2.787	4.286	33	5.175
25	63	63	03	47
26	42	44	3.575	25
27	21	21	48	5.097
28	03	03	24	75
29	2.684	4.185	00	52
30	66	66	3.476	30

S indicates the volume of yeast-glucose mixture from which CO_2 was extracted in the 50 cc. Van Slyke-Neill chamber. a indicates gas volume at which P_{CO_2} was measured.

The precautions concerning uniform timing of the analysis mentioned for the macromethod hold also for the micromethod.

Blank Analysis—This is performed with a sample of 0.2 per cent Na_2SO_4 in place of blood filtrate. The value obtained at the 0.5 cc. mark for $p_1 - p_0$ is taken as the c correction. The precautions necessary in determining the c correction in the macromethod apply here also.

Calculation—See the macroanalysis. The factors for micro-

analysis in Table I have been found valid for blood with sugar concentrations up to 400 mg. per cent (see Table VII).

EXPERIMENTAL

CO₂ Yield under Varying Conditions of Fermentation

The macromethod was applied to a standard solution of glucose containing 1 mg. in 5 cc., and the acid concentration was varied in order to note the effect of this factor on CO₂ yield. The results

TABLE II

Yield of CO₂ from 1 Mg. of Glucose with Varying Acid Concentration; Macro-analysis with Brewers' Yeast

Temperature	Acid concentration*	Pressure of CO ₂ from glucose, measured at 2 cc. volume	CO ₂ per mole glucose†
°C.	M	mm.	moles
26	0.04 tartaric acid	65.6	1.55
26	0.066 KH ₂ PO ₄	66.5	1.57
26	0.04 citric acid	72.0	1.71
25	0.02 " " in 0.4% NaCl	71.7	1.71
25	0.04 " " " 0.4% "	72.3	1.72
25	0.08 " " " 0.4% "	68.5	1.63
25	0.16 " " " 0.4% "	59.7	1.42

* This figure refers to the final concentration after the sample has been added. A higher concentration exists, of course, during the period of CO₂ removal from the yeast.

† In order to calculate moles of CO₂ per mole of glucose, the moles of CO₂ obtained in each analysis were found by means of Equation 3 of Van Slyke and Sendroy (1927), with $S = 10$ cc. Then

$$\frac{\text{Moles CO}_2}{\text{Moles glucose}} = \text{moles CO}_2 \times \frac{180}{\text{gm. glucose in sample}}$$

are shown in Table II. The optimum citric acid concentration lies at about 0.04 M. NaCl added to the citric acid as a preservative has no effect on fermentation.

In experiments with yeast suspended in dilute citric acid, the fermentive power of the yeast fell slowly, as noted by Lange (1907) (see Table III). In this connection it is of interest to note that Stavely, Christensen, and Fulmer (1935) found the optimum pH for CO₂ production by zymoin from brewers' yeast to lie between 5.8 and 6.2, approximately the pH reported by Mahdi-

hassan (1930) for the yeast cell interior. These facts would suggest that the deleterious effect of citric acid on yeast fermentation may be due to diffusion of the acid into the cells. In the method presented, this effect is reduced to constancy by adding the citric acid to the yeast at the beginning of each analysis.

Fermentation Time and Maximum CO₂ Yield

Fermentation has rarely been found to yield as much as the theoretical 2 moles of CO₂ per mole of glucose. Van Slyke and Hawkins (1929) obtained from 71 to 79 per cent of theoretical, using commercial bakers' yeast and 1 hour fermentation periods, with conditions devised for determining fermentable sugar in

TABLE III

Yield of CO₂ from 1 Mg. of Glucose with Varying Age of Yeast-Citric Acid Suspension, Macroanalysis

Fermentations were performed with 5 cc. portions of a suspension prepared by mixing 30 cc. of 1:2 yeast suspension with 20 cc. of 0.2 N citric acid in 2 per cent NaCl. Temperature 25°.

Time after preparing yeast-citric acid suspension	Brewers' yeast	Bakers' yeast
	CO ₂ per mole glucose	CO ₂ per mole glucose
min.	moles	moles
0	1 71	1 32
20	1 70	1 18
40	1 48	1 09
60	1 26	1 00

urine. Employing glucose concentrations of 0.04 to 0.08 per cent, Hopkins and Roberts (1935) obtained 77 per cent of theoretical CO₂ with pressed washed brewers' (top) yeast. Meyerhof and Schulz (1936) have raised the yield to 95 per cent by adding nitrogenous tissue extractives.

Under the conditions used in our analyses the maximum yield of CO₂ with brewers' yeast was 86 per cent of the theoretical, and was obtained in 3 minutes. Extending the time longer did not increase the yield of CO₂ from glucose. With bakers' yeast the maximum yield, 66 to 67 per cent, was obtained in 4 minutes

at 25–30°, and in 5 minutes at 20°. Data showing the rates of CO₂ evolution are given in Table IV.^a

TABLE IV

Rate of CO₂ Evolution from Glucose Fermented at 20°, 25°, and 30° under Conditions of Macro- and Microanalyses

Temperature	Fermentation time	CO ₂ evolved per mole glucose		
		Brewers' yeast	Bakers' yeast	
		Macroanalysis, 1 mg. glucose	Macroanalysis, 1 mg. glucose	Microanalysis, 0.184 mg. glucose
°C.	min.	moles CO ₂	moles CO ₂	moles CO ₂
20	1	0.47		0.50
	2	1.00	0.78	0.78
	3	1.72	0.94	1.16
	4	1.72	1.20	1.32
	5	1.73	1.32	1.33
	6		1.32	
25	1	0.52	0.51	0.52
	2	1.43	1.04	0.99
	3	1.72	1.27	1.15
	4	1.71	1.33	1.36
	5	1.72	1.33	1.37
30	1	0.53	0.68	0.67
	2	1.40	1.18	1.11
	3	1.72	1.27	1.35
	4	1.73	1.34	1.35
	5	1.73	1.34	1.36

Effect of Temperature on CO₂ Yield

Between 20–30° the effect of temperature change on the rate of CO₂ evolution is slight (almost undetectable with the brewers'

^a Slightly higher values were obtained with a fermentation technique in which the yeast was treated with 1.5 cc. of 20 per cent trichloroacetic acid at the end of the fermentation period in order to kill it and free it completely of CO₂. In this way the fermentation was 88 per cent of the theoretical, but the results obtained were otherwise entirely similar to those with the method outlined. Part of the data to follow were obtained by the trichloroacetic acid method, which gave satisfactory results although requiring more time for each analysis.

yeast), whereas if its pace were set by a chemical reaction, enzymic or other, one would expect the rate to be about doubled by a temperature rise from 20–30°. The absence of such a temperature effect suggests that the limiting velocity factor may be, not the fermentation reactions, but the speed of diffusion of glucose into the yeast cells; this in turn depends partly on the efficiency of stirring of the yeast suspensions, which does not vary with temperature. The unusual quickness of fermentation in our analyses is probably due to the facts, first that the yeast to glucose ratio used is high, and, second, that the efficient stirring of the rapidly shaken chamber achieves quick contact and passage of all the sugar molecules into the yeast cells, so that the zymase can get at once into action.

TABLE V

Effect of Temperature Changes Between 20–30° on CO₂ Yield

Each PCO_2 value is the mean of four analyses \pm the average deviation.

Temperature	Brewers' yeast Macromethod 1 mg. glucose, 3 min. fermentation		Bakers' yeast			
			Macromethod 1 mg. glucose, 4 min. fermentation		Micromethod 0.154 mg. glucose, 4 min. fermentation	
	PCO_2 at 2 cc. volume	CO ₂ per mole glucose	PCO_2 at 2 cc. volume	CO ₂ per mole glucose	PCO_2 at 0.5 cc. volume	CO ₂ per mole glucose
°C.	mm.	moles	mm.	moles	mm.	moles
20	69.0 \pm 0.1	1.71	53.1 \pm 0.2	1.32	37.0 \pm 0.1	1.32
25	72.0 \pm 0.1	1.715	55.5 \pm 0.2	1.32	39.0 \pm 0.2	1.35
30	74.9 \pm 0.2	1.72	58.2 \pm 0.2	1.34	39.9 \pm 0.1	1.35

The yield of CO₂ in moles per mole of glucose, as shown in Table V, was found to be almost constant in the range 20–30°.

CO₂ Yield from Varying Amounts of Glucose

A direct proportionality was observed between the yield of CO₂ and amount of glucose, so long as the latter did not exceed that found in determinations of blood sugar up to 300 mg. per 100 cc. by the macromethod, or 400 mg. per 100 cc. by the micro-method. With larger amounts of glucose, a longer fermentation period was required to attain a maximum CO₂ yield (see Tables VI and VII).

TABLE VI
CO₂ Yield from Varying Amounts of Glucose Fermented with Brewers' Yeast

Glucose fermented	Corresponding blood sugar concentration	Pressure of CO ₂ from glucose	CO ₂ per mole glucose
Macromethod; temperature 25°; 3 min. fermentation period			
mg.	mg. per 100 cc.	mm.	moles
0.25	50	18.5	1.76
0.25		17.9	1.71
0.50	100	36.6	1.74
0.50		36.3	1.73
1.00	200	72.7	1.73
1.00		72.9	1.74
1.00		71.1	1.70
1.00		71.4	1.70
1.00		72.1	1.72
1.00		72.0	1.72
1.00		72.9	1.74
1.00		73.3	1.75
1.50	300	106.0	1.69
1.50		106.2	1.69
2.00	400	132.2	1.58
2.00		132.1	1.57
2.00		131.8	1.57
2.00		131.8	1.57
Macromethod; temperature 25°; 5 min. fermentation period			
2.00	400	146.1	1.74
2.00		146.4	1.74
Micromethod; temperature 28°; 3 min. fermentation period			
0.077	100	23.8	1.63
0.077		23.3	1.60
0.154	200	47.4	1.63
0.154		47.5	1.63
0.154		47.6	1.63
0.154		47.9	1.64
0.308	400	96.3	1.65
0.308		95.8	1.64

*Comparison of Fermentable Sugar by Macromethod with That
by Somogyi Technique*

Values for fermentable blood sugar by the macromethod were found to agree closely with those obtained by the Somogyi

TABLE VII
CO₂ Yield from Varying Amounts of Glucose Fermented with Bakers' Yeast

Glucose fermented	Corresponding blood concentration	Pressure of CO ₂ from glucose	CO ₂ per mole glucose
Macromethod; temperature 25°; 4 min. fermentation period			
<i>mg.</i>	<i>mg. per 100 cc.</i>	<i>mm.</i>	<i>moles</i>
0.50	100	28.1	1.34
0.50		28.0	1.33
1.00	200	55.2	1.32
1.00		55.7	1.33
1.00		55.6	1.33
1.00		55.1	1.31
1.50	300	80.7	1.28
1.50		80.6	1.28
2.00	400	100.8	1.20
2.00		100.6	1.20
Micromethod; temperature 25°; 4 min. fermentation period			
0.077	100	19.5	1.35
0.077		19.5	1.35
0.154	200	39.1	1.36
0.154		39.0	1.35
0.154		39.0	1.35
0.154		39.1	1.36
0.231	300	57.3	1.33
0.231		57.6	1.33
0.308	400	69.7	1.21
0.308		69.9	1.21

(1927) fermentation-reduction technique in a series of patients, including some with marked nitrogen retention. The results

TABLE VIII

Comparison of Fermentable Blood Sugar (Brewers' Yeast) by Present Macro-method with Fermentable Sugar by Somogyi Technique

Patient No.	Reducing sugar, Somogyi			Fermentable sugar by direct CO ₂ measurement	Non-protein nitrogen
	Total	Non-fermentable	Fermentable		
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
1	100			95	71
2	76			76	72
3	120	5	115	113	130
4	173	7	166	168	97
5	120	8	112	114	200
6	77	0	77	79	
7	73	1	72	76	
8	73	0	73	73	
9	97	1	96	94	
10	83	0	83	80	
11	76			72	
12	66	2	64	65	
13	81	4	77	74	
14	92	1	91	94	
15	87	2	85	84	
16	72	1	71	71	
17	74	3	71	72	
18	79	0	79	79	
19	100	0	100	103	
20	107	0	107	109	
21	102	0	102	102	
22	82	0	82	83	
23	91	0	91	90	
24	78	0	78	79	
25	82	0	82	82	
26	74	0	74	71	
27	60	0	60	60	
28	100	0	100	102	
29	80	0	80	75	
30	89	0	89	84	
31	99	0	99	94	
32	78	0	78	75	
33	85	0	85	81	
34	71	1	70	69	

are given in Table VIII. No diabetic blood was available for examination. All of the determinations, both gasometric and reduction, were performed on Somogyi (1930) filtrates. 5 cc. samples of filtrate were used for each analysis. The Shaffer-Somogyi (1933) iodometric Reagent 50, containing 1 gm. of KI per liter, was used for the determination of reducing sugar.

Analysis of Whole Blood without Removal of Proteins

From a series of oxalated blood specimens, 3 cc. portions of each blood were diluted to 30 cc. in volumetric flasks with Somogyi

TABLE IX

*Fermentable Sugar Determined in Blood Filtrates and in Diluted Blood.
All Determinations by Gasometric Macromethod (Brewers' Yeast)*

Patient No.	Somogyi filtrates	Folin-Wu filtrates	Whole blood, diluted
	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
1	88	88	88
2	71	71	70
3	201	203	203
4	82	80	81
5	97	95	97
6	110	109	111
7	128	128	
8	84	85	85
9	82	82	77
10	143	144	145
11	82	82	86
12	81	78	81
13	99	102	108

(1930) and Folin-Wu (1919) reagents for protein precipitation. A third portion from each sample was diluted 10-fold with distilled water to which were added 6 drops of saturated citric acid solution.⁹ The macromethod was then applied to the two fil-

⁹ The determinations on diluted whole blood were troublesome, because an unusually long time was required to remove preformed CO₂, presumably on account of buffer action by the blood proteins. Trouble was also caused by foaming when the blood and yeast were shaken together. Caprylic alcohol cannot be used to prevent foaming, because it retards fermentation. The use of whole blood is not recommended for analytical work.

trates and the diluted blood. Fermentable sugar was calculated by the factors of Table I. The two filtrates and the diluted blood gave nearly the same results for each specimen of blood, indicating that non-glucose substances, even in whole blood, have little if any effect upon the CO_2 yield in the fermentation method. The results are shown in Table IX.

Effect of Glycolysis on Fermentable and Reducing Sugar of Blood

In order to compare the effect of glycolysis on the reducing material of blood with the effect on fermentable sugar measured by the present method, samples of blood were drawn from two patients. A portion of each sample was deproteinized with

TABLE X
Effect of Glycolysis at 25° on Reducing Sugar of Blood Compared with Fermentable Sugar by Macromethod

Time	Sample 1		Sample 2	
	Reducing sugar	Fermentable sugar	Reducing sugar	Fermentable sugar
hrs.	mg. per cent	mg. per cent	mg. per cent	mg. per cent
0	100	103	107	109
2	91	104	94	110
4	86	102	84	110
24	17	46	7	18

Somogyi (1930) reagents at once and analyzed for reducing sugar by the Shaffer-Somogyi (1933) iodometric Reagent 50 and for fermentable sugar by the macrogasometric method. The rest of the blood was allowed to stand at 20–25°, and comparative analyses were run at intervals of 2, 4, and 24 hours. The results are shown in Table X.

There was no appreciable change in the CO_2 evolved by yeast for the first 4 hours, during which time there was nevertheless a drop of 15 to 20 per cent in the reducing value. Both the reducing value and the fermentable sugar value fell off sharply by the end of 24 hours. From the difference during the first 4 hours, it appeared probable that the first products of glycolysis in blood, although possessed of diminished reducing power, may

yield as much CO_2 on fermentation as did the parent glucose. In this connection, the fermentability of 1 mg. samples of lactic acid

TABLE XI
Comparison of Fermentable Blood Sugar Values by Micromethod with Those Obtained by Macromethod (Brewers' Yeast)

Patient No.	Fermentable sugar calculated as glucose	
	Micromethod	Macromethod
	<i>mg. per cent</i>	<i>mg. per cent</i>
1	84	86
2	71	68
3	128	121
4	59	65
5	124	123
6	135	137
7	99	101
8	60	58
9	67	65
10	103	98

TABLE XII
Yields of CO_2 with Different Types of Yeast. Macromethod
Fermentation of 1 mg. of glucose at 25° .

Type of yeast	Pressure of CO_2	CO_2 per mole glucose
	<i>mm.</i>	<i>moles</i>
Sample 2019, fresh pressed, brewers' (Fleischmann)*	72.2	1.72
Sample 134, fresh pressed, bakers' (Fleischmann)*	56.4	1.34
Fleischmann's Yeast, bakers', flour cake	55.5	1.32
Active dry yeast (Fleischmann)	53.8	1.28
Brewers' Strain L (Anheuser-Busch)	50.3	1.20
Busch Yeast, flour cake	46.5	1.11
Sample 139, starch-free yeast (Fleischmann)	35.1	0.84
" 2019, dry	Negligible	
Harris dry brewers' yeast	"	

* Samples 2019 and 134, fresh pressed, are available only by special request. The active dry yeast is troublesome to use, because of foaming.

was tested by the macromethod with negative results. This would suggest that lactic acid may not be formed during the first stage of the glycolysis.

Comparative Analyses by the Micro- and Macromethods

The values obtained for fermentable sugar by the micromethod were found to agree well with those by the macromethod (Table XI).

Yield of CO₂ with Different Types of Yeast

The maximum CO₂ yields with several different types of commercial yeast are shown in Table XII.

SUMMARY

Methods are described for the direct estimation of fermentable blood sugar, which is measured by the CO₂ produced by yeast in the manometric apparatus of Van Slyke and Neill. Determinations can be completed in 6 minutes, and with as little as 0.12 cc. of blood.

The writer wishes to express his gratitude to Dr. Donald D. Van Slyke, Dr. Kenneth Goodner, and Dr. René Dubos for their helpful advice.

Addendum—An alternative method of timing the analyses, which may give more constant blanks, is to start the timing at the end of the preliminary 1½ minutes shaking, and allow a constant period (e.g. 4½ minutes) from then till the end of fermentation, such that the fermentation will receive approximately 3 minutes.

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THE METABOLISM OF AMIDES IN GREEN PLANTS*

I. THE AMIDES OF THE TOBACCO LEAF

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Although asparagine and glutamine have long been known as constituents of green plants, it is only in recent years that an explanation of the function of these amides has been advanced. Prianschnikow (1), in 1922, arrived at the conclusion that amides are formed in response to the presence of ammonia in the tissues in order to minimize the toxic effects of high concentrations of the ammonium ion. The close analogy with the function of urea in animal metabolism was emphasized in a later paper (2).

There is an extensive literature on the enrichment of the amide content of the tissues when plants are treated in a manner calculated to bring about an accumulation of ammonia. Etiolation of seedlings, which interferes with protein synthesis in the axial organs under conditions in which nitrogen is being rapidly supplied in soluble form from the digestion of the protein of the cotyledon, gives rise to rapid storage of asparagine or glutamine, depending on the species (3). Borodin (4) first observed that amides are also rapidly synthesized in leaves when these are "starved" in water culture, that is under conditions which permit unrestricted hydrolysis of the tissue protein (5, 6), and amides are also formed in plants when an excessive quantity of ammonium salts is applied to the roots (7, 8).

Although the close correlation of amide synthesis with the presence of ammonia is obvious, the nature of the other components

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essential to the formation of the specific substances asparagine and glutamine is by no means clear. That these two amides may arise directly from the tissue proteins by hydrolysis of peptide bonds is possible (9), but the quantities actually observed under many experimental conditions are far in excess of the quantities that could be provided from this source on any reasonable hypothesis of protein constitution; this was clearly pointed out many years ago by Schulze (10). It is necessary, therefore, to assume that most of the newly synthesized amide is formed by the reaction of ammonia with a non-nitrogenous precursor and, inasmuch as two amides are commonly encountered, there must be two of these. Differences in the metabolic relationships of the two amides are therefore to be expected.

The probability that the precursors of the amides are products of carbohydrate metabolism follows from the early work of Suzuki (7) and has been carefully examined by Prianischnikow (1, 11) and by Smirnow (12), but experiments designed to ascertain their nature more precisely have not been entirely successful. Mothes (13), proceeding on the assumption that the intermediate compound is an organic acid, has demonstrated amide synthesis in mature leaves into which the ammonium salts of malic, succinic, or fumaric acids were introduced by vacuum infiltration (14). Schwab (15) has repeated some of this work, however, and finds that, if sufficiently dilute solutions are used for the infiltration, there is no difference in the amount of amide synthesis whether ammonium malate, oxalate, or sulfate is used. If this is correct, the nature of the acid ion is immaterial, and consequently no genetic connection between the newly formed amide and the added organic acid can be inferred.

Our own experimental attack on the problem of amide metabolism has been chiefly intended to reveal relationships between protein decomposition and amide synthesis in leaves cultured in water. When excised tobacco leaves are placed with their bases in distilled water and are kept in the dark, a rapid hydrolysis of the leaf protein occurs with the corresponding production of an increase in the water-soluble nitrogen. An earlier study of the forms of combination of the soluble nitrogen enabled us to point out (6) that there is a close correlation between the increase in amide nitrogen and the decrease in the soluble amino nitrogen. At the

time of the previous work, we were not in a position to discriminate between asparagine and glutamine amide nitrogen, and, moreover, no attempt was made to investigate the effect of light on the transformations. The experiments have accordingly been repeated with attention to these matters. The detailed evidence upon which the following statements are founded is given in the experimental part.

When excised tobacco leaves are cultured in water, or in dilute glucose or nutrient salt solution in the dark, asparagine is rapidly synthesized. The increase in glutamine that occurs is very small, and is no more than can be accounted for on the assumption that that part of the leaf protein which undergoes digestion yields a proportion of glutamine by peptic hydrolysis of the order of magnitude that would be expected on the basis of Miller's (16) determinations of the glutamic acid yielded by leaf proteins on complete hydrolysis.

When cultured under otherwise similar conditions but in continuous light (artificial light at night), both glutamine and asparagine are synthesized. During the first 73 hours, the entire quantity of amides formed was approximately the same as the quantity of asparagine formed in the dark culture experiments; later, however, the total amide dropped behind the quantity formed in the dark. This behavior follows very closely the relative rates of digestion of the protein in the various experiments. Protein digestion proceeded at approximately the same rate regardless of illumination for the first 73 hours; subsequently the rate of digestion of the protein in the leaves in light diminished, while the rate in the darkened leaves was maintained. Although a strict correlation of the two phenomena is speculative, the data are in close agreement with the view that the part of the protein that was digested was converted into amino acids,¹ these were deaminized, and the ammonia produced was promptly utilized for the synthesis of an amide. The quantity of nitrogen involved in this series of reactions was in each case adequate to account for the whole of the amide nitrogen, and also a part of the amino nitrogen of the glutamine and asparagine. The deficiency in available ammonia

¹ Evidence that the digestion of leaf protein is complete to the amino acid stage has been previously presented (6). The highly active nature of leaf enzymes in undamaged tissue has also been emphasized by Paech (17).

necessary to complete the synthesis of the two amides may readily have been made up from the reduction of the nitrate present in the leaf tissue, and, in fact, when the quantity of nitrate that disappeared is added to the amino nitrogen presumably involved in these reactions, a remarkably close agreement is obtained with the total quantity of nitrogen ultimately combined in the form of glutamine and asparagine.

The synthesis of the amides can therefore be logically accounted for with respect to the quantity of nitrogen involved in the reactions, but with regard to the origin of the specific substances, whether glutamine or asparagine or both, very little has been ascertained. It is obvious that this is determined by the nature of the precursor which provides the carbon chain of the compound. It may be inferred that a precursor necessary for the formation of asparagine is present in normal tobacco leaves, which was drawn upon for the synthesis of asparagine in the dark. The quantity was inadequate to combine with all the ammonia that was produced inasmuch as considerable free ammonia accumulated towards the end of the culture period. It is also clear that the sequence of events is altered in the illuminated leaves. Here both amides were formed, and the quantity of glutamine, particularly towards the end of the culture period, exceeded the quantity of asparagine by from 50 to 100 per cent. No significant accumulation of ammonia occurred, and consequently it may be assumed that an excess of one or both precursors was present. The simplest assumption to account for the observations is that photosynthesis provided the precursor necessary for glutamine synthesis in the illuminated leaves.

That photosynthetic reactions played a large part during the culture of the leaves in light is evident from the rapid increase in organic solids that occurred. The extent to which new substances were elaborated is indicated by the fact that a kilo of fresh leaves, which contained 74.6 gm. of organic solids, after having been cultured in light in water for 235 hours, contained 110 gm. of organic solids, and only 16 gm. of the increased amount could be accounted for as reducing substances calculated as glucose. Less than 2 gm. of starch was present. Accordingly, if it be assumed that the reactions of photosynthesis ultimately result in the formation of a sugar, more than half of the newly formed

carbohydrate must have been further elaborated into substances which could no longer be demonstrated by the conventional reduction methods. Obviously the illuminated leaf tissues were the seat of a complex series of reactions of which the classical reaction of photosynthesis was only the preliminary step.

The most important problem of amide metabolism is at present the matter of the identification of the two precursors from which the carbon skeletons of asparagine and glutamine respectively originate. Granting the presence of sufficient of one or both of these, ammonia, whether of extraneous origin (*i.e.* from the culture solution) or produced endogenously from amino acid deamination, nitrate reduction, or otherwise, is promptly and efficiently cared for. In this connection Prianischnikow's idea of detoxication is illuminating and suggestive. But the nature of the precursors is still most obscure. All the evidence that has been obtained points to a carbohydrate origin for these substances and, as Prianischnikow and Schulow (11) have shown for asparagine and Vickery, Pucher, and Clark (8) for glutamine, there is direct evidence that they are non-nitrogenous.

Whatever the nature of the glutamine precursor may be, it, or a substance from which it is readily derived, is stored in considerable amounts in the root of the beet (8). The tomato plant can likewise synthesize this substance (18), and the old observations of Schulze (19) indicate that it is present in the sprouting seeds of the castor bean and pumpkin and in many other plants. The normal tobacco leaf contains very little, if any, but has the capacity to produce it under the action of light. When tobacco leaves in water culture are exposed alternately to light and to darkness for 24 hour periods, glutamine synthesis proceeds at a rate indistinguishable from that in continuous light. If the periods are 48 hours long, the rate is somewhat depressed. This suggests that a store of the precursor accumulates during each illumination period sufficient to care for glutamine synthesis during an ensuing 24 hours of darkness at undiminished velocity, but insufficient to care for a 48 hour period at the same rate.

The observations on the asparagine content of the same leaves show a somewhat analogous behavior. Asparagine is rapidly synthesized in the dark, slowly in the light. With alternations of light and dark, whether of 24 or of 48 hours duration, the rate of

asparagine synthesis is intermediate and is not materially different in the two series. Although the evidence is not conclusive, it suggests that if both precursors are present, glutamine is synthesized somewhat more abundantly than asparagine and, accordingly, a greater total glutamine synthesis takes place either in continuous light or with 24 hour alternations than of asparagine. It is possible that a mass action effect may determine the relative rates of formation of the two amides.

EXPERIMENTAL

The data from which the above conclusions have been drawn are derived from a series of culture experiments designed to obtain information with respect to the general behavior of the various groups of components of leaf cells. The technique has been fully described elsewhere (6) and the analytical methods employed were essentially those given in a recent bulletin (20) from this Station.²

In brief, a uniform series of samples of tobacco leaves picked from the same location on the plant was selected, each sample consisting of 60 leaves. Control samples were set aside for immediate analysis, and the others were placed with the bases of the leaves in water or nutrient solution. For the light experiments, the leaves were arranged in troughs so constructed as to give maximum exposure of the leaf surface, and were placed in a greenhouse; artificial light was supplied at night. For the dark experiments, the leaves were arranged in pails kept in a dark room and were examined only under dim red light. From time to time samples were withdrawn, a subsample was selected for analysis in the fresh condition, and the remaining leaves were dried at 80° in a rapid current of air (1 to 2 hours); the dry tissue, after being ground to a fine powder, was analyzed. The data are calculated in terms of gm. per kilo of original weight of the fresh tissue. If it be assumed that the initial composition of each sample was the same, the differences later found represent the magnitude of the changes that have occurred in each constituent and, when expressed in this way, are more readily apprehended than if a percentage method of calculation were used. Furthermore, inasmuch

² The complete data, together with a fuller discussion of the results than can be given here, will be presented in Bulletin 399 (21) from this Station.

as both the fresh weight and the dry weight are subject to considerable change, comparisons on a percentage basis may lead to incorrect conclusions.

Table I shows data for the organic solids in six experiments, three in the dark and three in the light, in three different culture solutions, distilled water (W), 0.36 per cent glucose (G), and a modified Tottingham solution (N) which contained ammonium sulfate as the source of nitrogen. The composition of this was KH_2PO_4 0.143, CaCl_2 0.233, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.875, and $(\text{NH}_4)_2\text{SO}_4$ 0.555 gm. per liter, and the solution was adjusted to an initial pH of 6.3 to 6.4 with potassium hydroxide. The letters D and L prefixed to the letter that shows the nature of the culture solution refer to dark or light culture. The last column shows the maximal

TABLE I
Organic Solids of Tobacco Leaves

The figures are given in gm. per kilo of original fresh tissue.

Culture	0 hr.	25 hrs.	73 hrs.	143 hrs.	235 hrs.	Δ
LW	74.6	78.8	95.1	100.8	110.3	+35.7
DW	74.6	70.2	66.1	60.0		-14.6
LG	88.6	100.3	107.3	100.5	101.2	+12.6
DG	88.6	86.9	77.0	66.2		-22.4
LN	76.2	88.1	86.2	91.9	97.8	+21.6
DN	76.2	81.2	73.9	72.5		-3.7

change in organic solids during the entire period studied. Synthesis of organic solids in light, and loss in dark culture are clearly evident, and the order of magnitude of the changes is surprisingly high. The three sets of leaves employed respectively for water, glucose, and salt solution culture were picked at intervals of 1 week, and were, in the same order, the first three leaves, the second three, and the third three leaves counting from the bottom of the plant. The three groups of leaves therefore differed somewhat in age and development.

The rate of protein digestion is shown in Table II which gives the data for the early part of the experiment in detail to illustrate the similarity in rate of digestion in the light and in the dark and the reduced rate of the digestion in the illuminated leaves in the later phases of the experiment. The figures are determinations of

the total nitrogen which remained insoluble after thorough successive extraction of the dried tissue with 75 per cent alcohol and with boiling water. Evidence that this nitrogen consists largely of protein nitrogen is given in Table III which shows the decreases in the quantities of amino and amide nitrogen of the protein contained in the extracted leaf residue. Each sample was subjected to complete hydrolysis with hydrochloric acid, and the amino

TABLE II
Protein Nitrogen of Tobacco Leaves

The figures are given in gm. per kilo of original fresh tissue.

Culture	0 hr.	25 hrs.	49 hrs.	73 hrs.	143 hrs.	235 hrs.	Δ
LW	2.27	2.13	1.92	1.75	1.59	1.56	-0.71
DW	2.27	2.09	1.98	1.71	1.00		-1.27
LG	2.35	2.75	2.27	2.27	1.79	1.29	-1.06
DG	2.35	2.45	2.34	2.07	1.20		-1.15
LN	2.61	2.81	2.32	2.31	2.08	1.89	-0.72
DN	2.61	2.54	2.44	2.17	1.45		-1.16

TABLE III
Decreases in Amino and Amide Nitrogen of Tissue Protein during Culture of Tobacco Leaves

The figures are given in gm. per kilo of original fresh tissue.

Culture	Amino N	Amide N	Ratio of amino to total N
	gm.	gm.	per cent
LW	0.52	0.045	57.6 \pm 0.5
DW	0.67	0.052	63.4 \pm 1.0
LG	0.69	0.039	61.3 \pm 0.6
DG	0.79	0.054	61.0 \pm 0.6
LN	0.51	0.049	59.9 \pm 2.2
DN	0.78	0.070	58.8 \pm 1.2

and ammonia nitrogen produced was determined. The figures in Table III show the difference between the value for the initial fresh leaf sample and the final sample in each culture experiment. The detailed data, when plotted, fall upon relatively smooth curves and are therefore omitted in the interests of economy of space. The average ratios of amino to total nitrogen of each set of determinations are given, together with the probable error, and the

order of magnitude is clearly that to be expected from the hydrolysis of protein.

Table IV gives a summary of the increases in the quantities of glutamine and asparagine amide nitrogen, and of the ammonia, and also of the decrease in the amounts of nitrate nitrogen which may be assumed to represent reduction of nitrate to ammonia. Attention has already been directed to the effect of illumination on the synthesis of glutamine; a comparison of the figures for apparent glutamine amide synthesis in the dark with the figures for the change in amide nitrogen of the protein, given in Table III, will serve to illustrate the statement that this quantity of glutamine may have arisen directly from peptic hydrolysis of the protein.

TABLE IV

Changes in Forms of Soluble Nitrogen during Culture of Tobacco Leaves

The figures are given in gm. per kilo of original fresh tissue.

Culture	Asparagine amide N	Glutamine amide N	Ammonia N	Nitrate N	Soluble amino N
LW	0.118	0.169	0.038	-0.198	0.600
DW	0.302	0.024	0.233	-0.277	0.562
LG	0.136	0.192	0.047	-0.214	0.687
DG	0.357	0.026	0.314	-0.281	0.617
LN	0.083	0.244	0.111	-0.122	0.756
DN	0.383	0.037	0.181	-0.060	0.727

The last column of Table IV gives the change in soluble amino nitrogen, the determinations in this case, as well as the determinations of free ammonia, having been made on water extracts prepared from a subsample from each lot of leaves which was withdrawn before the main sample was dried. The subsamples were treated with ether to cytolyze the cells, and were then thoroughly extracted with cold water with use of the hydraulic press, according to the technique of Chibnall (22).

The calculation of the amino nitrogen which disappeared as such can be made from the data in Tables III and IV. The water-soluble amino nitrogen is obviously in part due to the amino nitrogen of asparagine and glutamine. Asparagine reacts normally in the Van Slyke apparatus to give half its nitrogen as amino nitrogen, but glutamine reacts abnormally to yield 90 per cent of

its total nitrogen under standard conditions (23). Thus, to correct the soluble amino nitrogen of the tissues for the amino nitrogen due to the amides, it is necessary to subtract the sum of the asparagine amide nitrogen and 180 per cent of the glutamine amide nitrogen; the difference then represents the amino nitrogen of amino acids other than these two amides. In the LW experiment this quantity is $0.600 - (0.118 + 0.304) = 0.178$ gm.

The amino nitrogen produced by the hydrolysis of protein during the culture period can be estimated from the data in Table III; it is clear that the difference between the amino nitrogen which can be liberated by complete hydrolysis of the residual protein at the termination of the culture period and that at the beginning represents amino nitrogen that must have been set free by the normal proteolytic enzymatic reactions of the tissue during the interval. Accordingly one would expect to find an increase of 0.52 gm. of amino nitrogen (Table III) in soluble form after the entire period of culture in the LW experiment. Actually the increase, due solely to amino acids other than asparagine and glutamine, is 0.178 gm. and thus the difference between these quantities, or 0.34 gm., represents the amino nitrogen that has disappeared as such. If it be assumed that this nitrogen was converted to ammonia by oxidative deamination, the ammonia may have in turn given rise to the synthesis of amides. A comparison of the quantities calculated on these assumptions is given in Columns 2 and 3 of Table V, and it is clear that in all save one case (LN) the amount of amino nitrogen which may have been metabolized is in excess of the amide nitrogen produced.

If, however, other sources of ammonia are considered, a fairly close account of the metabolic changes may be given. The reduction of nitrate is such a possibility, and if the loss of nitrate nitrogen is added to the loss of amino nitrogen (Column 4), a quantity is obtained which may be designated the "metabolic ammonia." If the nitrogen combined as asparagine and glutamine is actually derived from metabolic ammonia, then the amide nitrogen multiplied by 2 should be of the same order of magnitude as this quantity. Reference to Columns 4 and 5 shows that this is true in the LW and LG experiments. But another factor must be considered in the experiments on dark culture inasmuch as free ammonia accumulated in these leaves in appreciable amounts.

Column 6 of Table V shows the sum of twice the amide nitrogen and the free ammonia in each case. It is this quantity which must be accounted for, inasmuch as it represents the total amide synthesis and the residual uncombined ammonia. The discrepancies between the figures in Columns 4 and 6 are shown in Column 7. Only two of the first five values exceed 0.1 gm., and when the possibilities of analytical and sampling errors are considered, it is clear that the agreement is all that could be expected.³

The effect of culture in a solution that provided an extraneous source of ammonia is shown in the last two values in Column 7. Not only was amide synthesis a little higher in each case than in

TABLE V

Relation between Amide Nitrogen Gain and Sources of Ammonia

The figures are given in gm. per kilo of original fresh tissue.

Culture	Amino N loss	Amide N gain	Nitrate N loss + amino N loss	Amide N \times 2	Amide N \times 2 + free ammonia N	Δ (6)-(4)
(1)	(2)	(3)	(4)	(5)	(6)	(7)
LW	0.341	0.287	0.539	0.574	0.612	0.073
DW	0.449	0.326	0.729	0.652	0.885	0.156
LG	0.483	0.328	0.697	0.656	0.703	0.006
DG*	0.500	0.311	0.665	0.622	0.734	0.069
	0.603	0.353	0.887	0.706	1.020	0.133
LN	0.275	0.326	0.397	0.652	0.763	0.366
DN	0.501	0.414	0.474	0.808	0.999	0.525

* The values for changes at 143 and 190 hours are given for the DG experiment.

the water culture experiment, but the accumulation of free ammonia was strikingly high, and for some reason that is not apparent the reduction of nitrate was low. Consequently the metabolic ammonia falls far short of the sum of the nitrogen present in the amides and as free ammonia, and it would appear that a quantity of ammonia of the order of magnitude of 0.4 gm. had been taken up from the nutrient solution.

³ No account is taken in this calculation of the soluble peptide nitrogen of the tissues nor of the small amounts of glutamine and asparagine which may have arisen directly from the protein by peptic digestion. Both were very small and the corrections, if introduced, would have no effect upon the conclusions.

Table VI shows the effect of alternation of light and dark on the synthesis of glutamine and asparagine in tobacco leaves. The samples used in this experiment consisted of the bottom three leaves of the plants and were cultured in distilled water. The light and dark control samples reproduce the phenomena already described under the experiments designated LW and DW above. The leaves of the dark control had become completely yellow and were in very bad condition at the expiration of 172 hours, and the analytical values are enclosed in parentheses to denote that the conditions were such that these samples could no longer be re-

TABLE VI

Effect of Alternation of Light and Dark on Amide Synthesis in Tobacco Leaves in Water Culture

The figures are given in gm. per kilo of original fresh tissue.

	0 hr.	24 hrs.	72 hrs.	120 hrs.	172 hrs.	240 hrs.
Glutamine amide						
Dark control.	0.008	0.014	0.068	0.063	(0.016)*	
Light "	0.008	0.029	0.128	0.154	0.178	0.213
24 hr. alternation.....	0.008		0.114	0.164	0.186	0.221
48 " "	0.008		0.068	0.101	0.139	0.106
Asparagine amide						
Dark control.	0.015	0.021	0.160	0.233	(0.143)*	
Light "	0.015	0.018	0.026	0.065	0.061	0.115
24 hr. alternation.....	0.015		0.036	0.109	0.108	0.146
48 " "	0.015		0.075	0.081	0.134	0.177

* Leaves in bad condition; no longer regarded as a control.

garded as a control upon the other values at this time. The same fresh leaf samples served as a point of departure for all series and the alternations of light and dark were so arranged that the 24 hour experiment was started in light, the 48 hour experiment was given a preliminary 24 hours in the dark. The data show that 24 hour alternations had no effect upon glutamine synthesis as compared with continuous illumination, but 48 hour alternations definitely retarded it. Alternation of light and dark stimulated a greater asparagine synthesis than was observed in continuous light, but the values did not approach those attained in

continuous dark. In those leaves in which both amides appeared the quantity of glutamine amide nitrogen exceeded the quantity of asparagine amide nitrogen with only one exception.

SUMMARY

When leaves of the tobacco plant are cultured in water or dilute nutrient solutions in the dark, a prompt and extensive enrichment in asparagine occurs. The simultaneous enrichment in glutamine is very small. On the other hand, when the leaves are exposed to continuous light, both glutamine and asparagine are synthesized, the former to a somewhat greater extent than the latter. Appreciable quantities of ammonia accumulate in the leaves subjected to dark culture, but the quantity in the leaves cultured in light is insignificant even after 235 hours.

It is shown that all of the nitrogen involved in the synthesis of the amides and in the production of free ammonia can be accounted for on the assumption that the amino acids that resulted from digestion of the protein during the culture period suffered deamination with the production of ammonia, and that the diminution in the nitrate content which likewise occurred also gave rise to ammonia. To account for the synthesis of the amides, it is then further assumed that a non-nitrogenous precursor of asparagine is present in normal tobacco leaves in sufficient amounts to give rise to the formation of a considerable quantity of this amide, and that, in addition, a non-nitrogenous precursor of glutamine is synthesized in even larger relative proportion when the leaves are exposed to light.

The nature of the non-nitrogenous precursors of the amides is unknown; the one required for the synthesis of glutamine is, however, produced by photosynthesis and is, accordingly, a carbohydrate or a metabolite of a carbohydrate. Glutamine then, and probably also asparagine, form important links between the general carbohydrate and protein metabolism.

The behavior of these two substances in the tobacco plant is interpretable in terms of Prianischnikow's hypothesis that amides are synthesized in plants in response to an increase in ammonia, and provide a mechanism whereby the ammonia concentration is ordinarily maintained below a toxic level. The tobacco plant furnishes an example of a species in which, under certain conditions, both amides are thus employed.

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CYSTINE METABOLISM

II. DETOXICATION OF MONOBROMOBENZENE

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Comparison of the results of the ultimate analysis of certain sulfur-containing proteins with the total quantity of sulfur amino acids present, as determined by specific methods, shows a discrepancy which in the case of casein and phaseolin is quite large.

This discrepancy, coupled with the substantial growth obtained by the authors (1) upon feeding rats a low cystine diet in the form of the navy bean diet of Finks and Johns (2), led us to speculate as to the presence of other sulfur-bearing amino acids.

Confirmation of this speculation was sought in the detoxication of monobromobenzene by cystine and similar substances.

In this respect White and Lewis (3) showed the detoxication of bromobenzene to take place by two paths, dependent upon the amount of cystine present. When a low cystine diet was used, the bromobenzene was detoxicated through conjugation with sulfuric acid and excreted in the urine as ethereal sulfates, with a corresponding decrease in the inorganic sulfate fraction. When an amount of cystine in excess of that required for maintenance of the animal was contained in the diet, the mechanism of detoxication differed and resulted in the excretion of *p*-bromophenylmercapturic acid:

More recently White and Jackson (4) caused a retardation of growth in young rats by incorporating bromobenzene in a diet in which the protein was supplied in the form of casein. They found both cystine and methionine to alleviate the deficiency.

In the light of these facts, the addition of a limited amount of bromobenzene to the bean diet should cause a retardation and possibly a cessation of growth. Further additions of bromo-

benzene should have little effect upon the weight and appearance of the animals until all of the sulfuric acid available for detoxication has been used up. Increasing the level of bromobenzene still further should cause serious tissue breakdown and other disturbances such as interference with digestion due to the non-reproduction of taurocholic acid and other important sulfur-containing compounds.

If cystine is the only detoxicating substance present, a corresponding level of bromobenzene will be reached beyond which growth will cease. If there are additional substances present, a higher level of bromobenzene will be tolerated before causing cessation of growth.

If the detoxication reaction went to completion, 2 moles of bromobenzene would detoxicate 1 mole of cystine, a ratio of 1.307. Then 100 gm. of bean meal, containing 45.82 mg. of cystine, would detoxicate 59.8 mg. of bromobenzene. The diet used contained 70 per cent of this bean meal.

EXPERIMENTAL

The growing rats of Series A and B previously reported (1) to be gaining on an average of 0.322 gm. and 0.260 gm. daily, respectively, were used for this experiment. The rats of Series A were maintained upon Diet 1, consisting of the Finks and Johns (2) basal, navy bean diet. Series B received the same diet supplemented with 2 per cent *l*-cystine and designated as Diet 2.

To these diets, fed *ad libitum*, were added increasing quantities of bromobenzene for various periods ranging from 10 to 40 days, during which time the weight and general condition of the rats in the respective series were recorded and used as a measure of the effect of the added bromobenzene and the extent of its detoxication; this is summarized in Table I.

The initial quantity of bromobenzene added corresponded to one-half the amount necessary to detoxicate the cystine known to be present in Diet 1. The effect, a slight decrease in the rate of growth of Series A, is given in Table I, in terms of the average daily change in weight. Periods 3, 4, and 5 correspond to intervals during which the concentration of bromobenzene was increased 2, 4, and 8 times. The result at twice the original concentration seemed to indicate the detoxication reaction to be quantitative,

inasmuch as a noticeable loss in weight was obtained; this effect was lost, however, as further increases in bromobenzene resulted both in small gains and losses. The concentration of bromobenzene added throughout Period 6, corresponded to the amount of bromobenzene that could be detoxicated if all the sulfur present in the diet were used for this purpose. The concentrations of bromobenzene used in Periods 7, 8, and 9 correspond to 5 per cent increases over the previous concentrations used. The concentrations of bromobenzene used in Periods 10, 11, and 12 correspond to 100, 200, and 300 per cent increases in concentration over that

TABLE I
Effect of Added Bromobenzene and Extent of Its Detoxication

Period No	Days on diet	Bromobenzene in diet	Series A change in weight	Series B change in weight
		<i>per cent</i>	<i>gm.</i>	<i>gm.</i>
1	180	0 0000	+0 322	+0 260
2	40	0 0209	+0 205	+0 405
3	25	0 0418	-0 044	-0 200
4	10	0 0836	+0 400	-0 025
5	10	0.1672	+0 400	+0 825
6	10	0 4970	+0.100	+0 350
7	10	0 5218	-0 420	+0 075
8	15	0.5466	+0 000	-1 500
9	20	0 5714	+0 180	+1 490
10	10	0 9940	+0 600	+1 200
11	15	1.4910	-0 400	+0 170
12	15	1.9880	+0 010	-0 200
13	15	0 0000	+1 850	+1 000

used in Period 6. The final values given in Table I are the result of placing the animals on our standardized complete rat ration to which no bromobenzene was added. The average growth curves throughout the entire experiment are given in Fig. 1.

The bromobenzene used was the redistilled product of the Eastman Kodak Company, and was of the highest quality. Experiments proved there was no loss due to evaporation of this product from the animal food pans.

Analysis of the results obtained shows no definite quantitative relationship between the concentration of cystine present in the

diet and the detoxication of bromobenzene over a very wide range of concentration. There is no evidence of a quantitative relationship between the amount of other sulfur compounds present and the detoxication reaction. The feeding of very high concentrations of bromobenzene indicates that the rat has means of detoxicating phenolic substances other than through its sulfur bodies. In fact this portion of the experiment casts doubt upon the premise that bromobenzene is at all toxic. The slight differences between the results obtained with the cystine-supplemented and

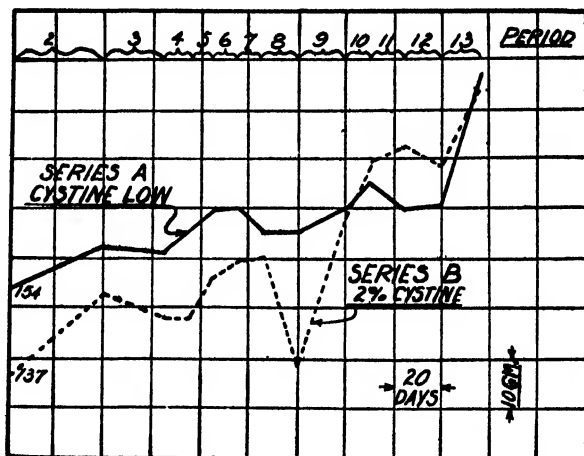


Fig 1 Average growth curves of rats on the low cystine diet and that with 2 per cent cystine

the unsupplemented diet (Diet 1) is further evidence of the non-quantitative nature of the detoxication reaction.

SUMMARY

A slow, variable rate of growth was obtained on adding various concentrations of bromobenzene to both a cystine-supplemented diet and an unsupplemented diet.

No evidence of the amount of cystine or similar sulfur compounds was detectable, and our speculation as to the presence of other sulfur compounds in phaseolin is unsupported.

Bromobenzene was tolerated at approximately a 2 per cent level,

which indicates this compound not to be particularly toxic to the rat.

The prolonged retardation of growth as a result of feeding a low cystine diet plus bromobenzene does not cause the rat to lose the capacity to grow when fed a normal diet.

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THE MICRODETERMINATION OF CHLORIDES IN BIOLOGICAL MATERIALS

PRESENTATION OF A METHOD AND AN ANALYSIS OF ITS USE

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In the method the Volhard reaction and the open Carius digestion of Laudat (1917) are used. The microvolumetric measurements are made with the precision syringe of Krogh and Keys (1931) and the Rehberg burette (1926).

Reagents and Apparatus—

Ammonium thiocyanate, NH_4CNS , approximately 0.1 N (0.76 gm. in 100 ml.).

Silver nitrate, AgNO_3 , approximately 0.2 N (34 gm. in 1 liter). Keep in a dark bottle.

Ferric alum, $\text{Fe}_2(\text{SO}_4)_3 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$. Dissolve 40 gm. in 100 ml. of hot water, allow to cool, and add 5 ml. of concentrated nitric acid.

Ether, chloride-free.

Hydrogen peroxide, 30 per cent (superoxol or perhydrol).

Sodium or potassium chloride, standard solutions 0.1, 0.8, and 0.5 N. Prepare from recrystallized, dried salts.

Syringe-pipettes¹ (Krogh and Keys, 1931), illustrated in Fig. 1,A. These greatly improve the ease and accuracy of the procedure. One, for blood, red cells, plasma, etc., should be set at about

¹The syringe-pipettes, with glass or steel needles to fit, and the Rehberg microburette may be obtained from the Workshop of Professor August Krogh, Juliane Maries Vej 32, Copenhagen, Denmark. Excellent Rehberg microburettes may also be obtained from the Macalaster-Bicknell Company, Washington and Moore Streets, Cambridge, Massachusetts. The reaction tubes (centrifuge titration tubes) and covers may also be obtained from the Macalaster-Bicknell Company.

0.2 ml.; another, which can be used for both urine and silver nitrate, should be fitted with a glass needle and set at about 0.5 ml.

Reaction tubes and covers¹ (centrifuge titration tubes), illustrated in Fig. 1, *B*. They are made of strong Pyrex glass. A long taper to the cone and a constant length from the constriction to the bottom are desirable. The flat bottom enables the covers to be used as microbeakers.

Rehberg microburette.¹ The 0.2 ml. size is convenient.

Measurement with Syringe-Pipette—The syringe-pipette delivers a constant volume (\pm about 0.0001 ml.) of liquid independently of viscosity (to about 20 times that of water) and temperature (between about 15–30°). It also contains a constant amount, but there is an appreciable dead space which must be flushed out when different liquids are delivered in succession.

Procedure A—When a number of deliveries of the same liquid are to be made, the syringe-pipette is flushed with the liquid three or four times, and inverted when necessary to expel all air bubbles. Deliveries are then made in succession without flushing; in each case 20 to 30 seconds are taken to fill and deliver and to pick off the last drop against the surface of the delivered liquid. Minute air bubbles which remain in the syringe do not interfere.

Procedure B—When only single or duplicate measurements of a liquid are to be made, the waste of liquid in preliminary flushing may be avoided by the following procedure which is generally used when measuring samples for analysis. The syringe-pipette is flushed with distilled water several times and discharged so that no air bubbles are left in the dead space. The end of the needle is touched against a clean surface to remove the last droplet and the syringe-pipette is then filled with the sample (the needle should be withdrawn slowly from the sample so that none of the sample adheres to it). The syringe-pipette is discharged into the appropriate reaction vessel, the last drop being picked off as usual, and is then flushed out with four or five changes of distilled water, all the washings being added to the reaction vessel. This entire procedure takes about 1 minute and the syringe-pipette is then ready for the next sample unless there is air in the dead space; if air is present, refill with water, invert and discharge the bubbles, and continue as with the first sample. Both the relative and the absolute accuracy of this procedure have been checked many times.

For many purposes it is unnecessary to know the exact delivery volume of the syringe-pipette. Calibration is easily carried out, however, by weighing the delivery of water. The weighing vessel should always be tared with a similar vessel containing water offering about the same surface for evaporation as in the weighing vessel. Alterations in the delivery volume from 0.1 to 1.6 ml. are readily made by means of the adjusting screw. When not in use the syringe-pipettes are always stored full of water and the plunger is never greased.

Procedure for Analysis—The samples are measured into centrifuge titration tubes by means of a syringe-pipette. For most purposes 0.2 ml. is convenient with Procedure B above. With the same syringe-pipette two standard chloride solutions of different concentrations are measured in duplicate into centrifuge titration tubes.

After all the chloride samples have been measured out, one delivery of silver nitrate solution is added to each centrifuge titration tube from the 0.5 ml. syringe-pipette, according to Procedure A above. The tubes are agitated briefly and 1 ml. of concentrated nitric acid is added to each so that the side of the centrifuge titration tube is washed down. The tubes are again agitated briefly and then stored in the dark until ready for digestion.

Digestion is carried out by heating in a bath of boiling water. As soon as the tubes have been brought up to the bath temperature 2 drops of H_2O_2 , 30 per cent, are added to each tube, including the standards. Plasma and whole blood are digested completely in less than 30 minutes at 100° . In the case of cells, however, digestion should be continued for at least 1 hour; at the end of the first 30 minutes 2 more drops of H_2O_2 are added (*cf.* below).

After digestion the tubes may be stored, protected from light (*cf.* below) and dust, as long as desired before titration. When ready for titration all the tubes are centrifuged for 10 to 15 minutes at a moderate speed. This packs all the AgCl precipitate in the bottom of the tube where it is effectually out of the sphere of titration, thus both clearing the field for recognition of the end-point and, perhaps more important, preventing the back reaction of AgCl with CNS^- (Keys, 1931).

Each tube receives a standard amount (1 or 2 drops of reasonably

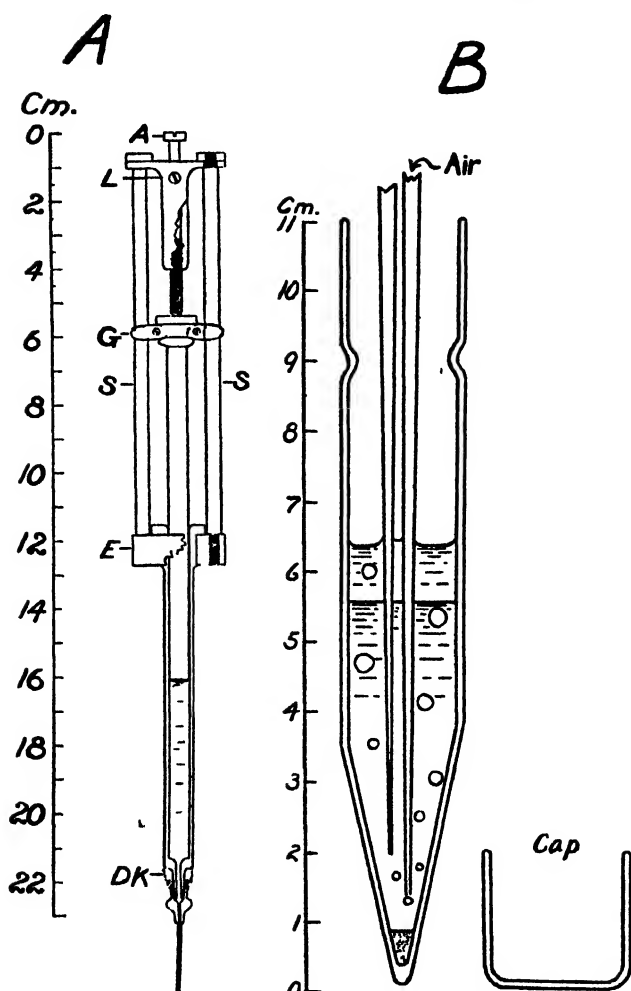


FIG. 1, A. Syringe-pipette, according to Krogh and Keys (1931). The volume of the delivery is adjusted by the screw A which is locked in position by the set screw L. The steel used for the support rods SS is selected so as to provide an automatic compensation for changes in temperature; i.e., the delivery is constant in amount in the temperature range 15–30°. The plunger of the syringe is moved by grasping the hard rubber plate, G, and the syringe itself is held by the hard rubber collar, E. The hypodermic needle is fastened to the syringe by cement, DK.

FIG. 1, B. Centrifuge titration tube and cap. The tube is shown as in use during a chloride titration.

constant size) of the concentrated ferric alum solution and, immediately before titration, 1 ml. of ether. During titration the AgCNS precipitate is collected at the interface between the ether and the watery solution so that the titration field is kept clear and back reactions are reduced to a minimum.

Titration is made with the usual Rehberg procedure; the delivery tip of the burette dips directly into the solution and stirring is done by an air jet (see Fig. 1,B).

Calculation—The calculation of the results may be made by the formula,

$$\text{Cl (m.-eq. per liter)} = 1000 \times \frac{(\text{volume} \times \text{concentration of AgNO}_3) - (\text{volume} \times \text{concentration of NH}_4\text{CNS})}{\text{volume of sample}}$$

where volumes are measured in ml. and concentrations of AgNO₃ and NH₄CNS in gm. moles per liter.

We much prefer, however, to use a graphical calculation from two chloride standards. Fig. 2 shows a sample calculation for plasma and whole blood with 0.08 and 0.1 N Cl standards.

Digestion—The use of 30 per cent hydrogen peroxide has none of the drawbacks that characterize Laudat's (1917) permanganate procedure, and yet the time required for complete digestion is very short.² After 20 minutes at 100° the chloride recovery from plasma and whole blood is about 99 per cent, and even with cells, better than 96 per cent complete. Table I shows the results of some experiments in which the time of digestion was varied. It will be seen that in all cases a maximum of 1 hour for plasma and whole blood and 2 hours for cells will suffice. If frothing is troublesome with cells or tissues, a drop of caprylic or octylic alcohol may be added to each tube (including standards).

The procedure advised here for cells will also suffice for many soft tissues. With samples of tissue containing much connective tissue or fat 4 to 8 hours may be needed to get complete digestion.

² In spite of statements to the contrary (e.g. Whitehorn (1920-21)), we have consistently found that digestion with only nitric acid does not give complete recovery of chloride unless nearly an hour at 100° is allowed for plasma.

The addition of the 30 per cent H_2O_2 at hourly intervals will accelerate the digestion.

Effect of Light—All papers on methods in which use is made of the Volhard titration contain specific instructions about the danger of exposure of the samples to light after the addition of the AgNO_3 . None of the descriptions of modern methods, however, shows any indication that the effect of light has actually been studied. We have examined this point in the following way: A

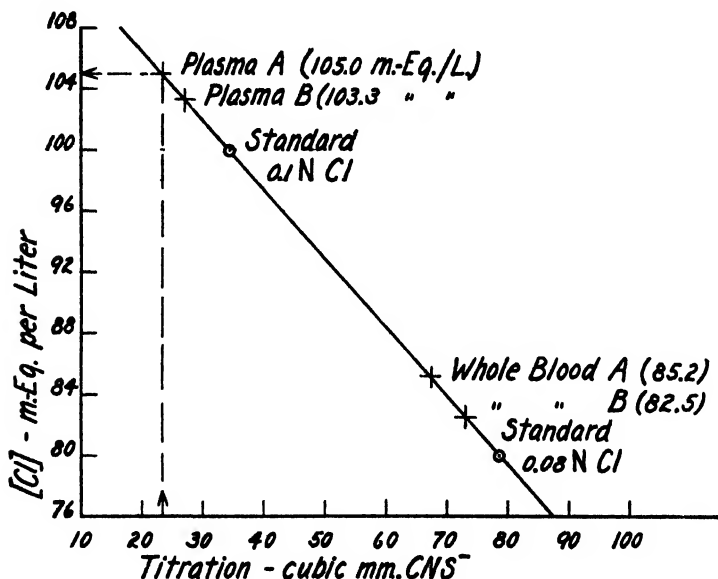


FIG. 2. Illustration of the graphical method of calculation of chloride concentration. Note that two standard chloride solutions are used and that the concentrations of the CNS^- and the Ag^+ solutions are neither known nor calculated.

large series of plasma samples was set up and digested in the manner described as standard for the present method. The series was divided into batches of four samples each, one batch being stored in the dark throughout, the other batches being left on the bench in the light of a north window for varying periods of time. No effect, as judged by the titration results, was detectable in 8 hours in the ordinary laboratory light when the excess Ag^+ for titration was relatively small (cf. Table II).

Obviously, the danger of exposure to light has been overemphasized. If a large excess of Ag^+ is used and prolonged exposure to direct sunlight is allowed, significant errors may arise, but even

TABLE I

Effect of Digestion Time on Apparent Chloride Concentration in Human Plasma, Whole Blood, and Red Cells

The digestion was carried out at 100° , with H_2O_2 , 30 per cent. The mean values, in milli-equivalents per liter, are from quadruplicate determinations in all cases.

Material	Digestion time					
	20 min.	40 min.	60 min.	120 min.	180 min.	240 min.
Plasma A.....	104.6	105.1	105.2	104.9		
" B.....	102.6	102.8	103.3	103.2	103.3	
Whole Blood C.....	82.5	82.3	82.7	82.4		
" " D.....	81.3	81.7	81.8	81.2	81.7	
Red Cells E.....	53.7	54.0	54.6	55.2	55.2	
" " F.....			55.3	55.3	54.9	55.2
" " G.....			58.20	58.92	59.02	58.92
" " H.....	56.1	56.6	57.2	57.7	58.1	57.9

TABLE II

Effect of Light on Apparent Chloride Concentration in Normal Human Blood Plasma

The samples were exposed, in Pyrex tubes, after precipitation of AgCl and digestion, to about 80 foot-candles of north daylight. The excess of Ag^+ for titration was equal to 14 per cent of the chloride content. The values of $[\text{Cl}]$ are given in milli-equivalents per liter.

Dark	2 hrs. light	5 hrs. light	8 hrs. light
103.00	103.04	103.12	102.80
102.92	103.00	102.64	103.08
103.20	102.72	103.00	103.32
103.00	102.64	102.72	102.80
Mean . . . 103.03	102.85	102.87	103.00

in this case, only when the standard chlorides are treated differently from the unknowns.

Storage of Prepared Samples before Titration—Frequently it is convenient to accumulate samples for chloride analysis until a

large number can be run through digestion and titration procedures together. There are two ways in which this can be done: (1) The samples may be measured out into the centrifuge titration tubes, and stored in the dark without the addition of AgNO_3 and HNO_3 ; (2) the samples can be measured out, the AgNO_3 and HNO_3 added, and the tubes stored in the dark completely ready for digestion.

In the first case, if the Cl standards are set up at the time the AgNO_3 and HNO_3 are added (just before digestion), the results are not in the least inferior to those obtained in the procedure in which no storage period intervenes.

In the second case, *i.e.* where the sample is stored with the AgNO_3 and HNO_3 , it might be expected that more difficulty would be met. Actually, storage up to at least 4 days, either at room

TABLE III

Effect of Storage of Samples of Blood Plasma, Plus Silver Nitrate and Nitric Acid, before Digestion and Titration for Chloride

Mean values, from determinations in triplicate, given in milli-equivalents of Cl per liter.

Species	No storage	48 hrs. at		96 hrs. at	
		0°	25°	0°	25°
Dog.....	107.5	107.4	107.7	107.5	107.4
Man.....	103.1	103.4	103.1	103.0	103.2

temperature or in the refrigerator, seems to do no harm. Experiments on the effect of storage are tabulated in Table III.

Centrifugation and Use of Ether—Most who have used the Volhard titration have warned against the danger of back reactions involving the AgCl and AgCNS precipitates. Numerous filtration procedures have been recommended to avoid these difficulties as well as to provide a clear field for the recognition of the end-point of the titration (*cf.*, *e.g.*, Rosanoff and Hill (1907)). However, except with extraordinary precautions, such filtrations are apt to introduce greater errors than they prevent, especially with microanalyses. In any case they much increase the labor involved.

Whitehorn (1920-21) showed that the Volhard titration in the presence of precipitates of silver salts is much improved if there

is a large excess of HNO_3 present. Results equal to those with the best filtration technique are readily obtained by the simple expedient of removing the AgCl from the sphere of the titration by brief centrifugation and titration of the supernatant fluid without removal from the reaction tube (Fiske and Sokhey, 1925; Keys, 1931). The omission of any separation of the precipitate from the titrated fluid results in values for chloride which are slightly too high. For example, a standard chloride solution, 94.80 milli-equivalents per liter, was analyzed in a lot of eighteen samples with the centrifugation-ether procedure; the mean value obtained was 94.87, probable error = ± 0.12 . When centrifugation was omitted (but ether was used), the mean value was 95.26 milli-equivalents per liter, probable error = ± 0.10 .

Rothmund and Burgstaller (1909) showed the utility of ether in the Volhard titration (see also Rehberg (1926)). We have not found any *mean* difference when ether is omitted (all the rest of the procedure kept constant), but the variability of separate determinations was increased about 3-fold. In addition, all technicians who have tried the omission of the ether have commented on the relative difficulty of judging the end-point.

Rate of Titration and Recognition of End-Point—The method of stirring by a current of air provides very rapid but not complete and instantaneous mixing. Moreover, the precipitation of the AgCNS requires an appreciable amount of time. Theory and experience combine to lead to the conclusion that it is best to titrate at a fairly uniform rate, reading the end-point in not less than 1 (better $1\frac{1}{2}$) minute. If the titration requires over 0.1 ml., 2 minutes should be taken.

When titration is not carried out too rapidly, the approach of the end-point is heralded by the final clearing of all cloudiness from the reaction mixture. For the end-point we prefer to take the first perceptible definite blush of rose color. This is persistent for about half a minute; owing to the excellence of the air stirring and the absence of finely dispersed AgCNS , a very transient end-point (*cf.* Osterberg and Schmidt (1927)) does not appear. After reading the burette at the end-point we add 1 c.mm. of CNS ; the resulting intense color is definitive.

Accuracy of Method—The accuracy of the method may be judged from (1) the agreement between duplicates; (2) the variability in

results from a long series of analyses on identical material; (3) the recovery of added chloride; (4) the agreement with the most refined macroanalysis; (5) the prediction of whole blood chloride concentration from separate analyses of plasma and cells (together with an accurate measure of the cell volume).

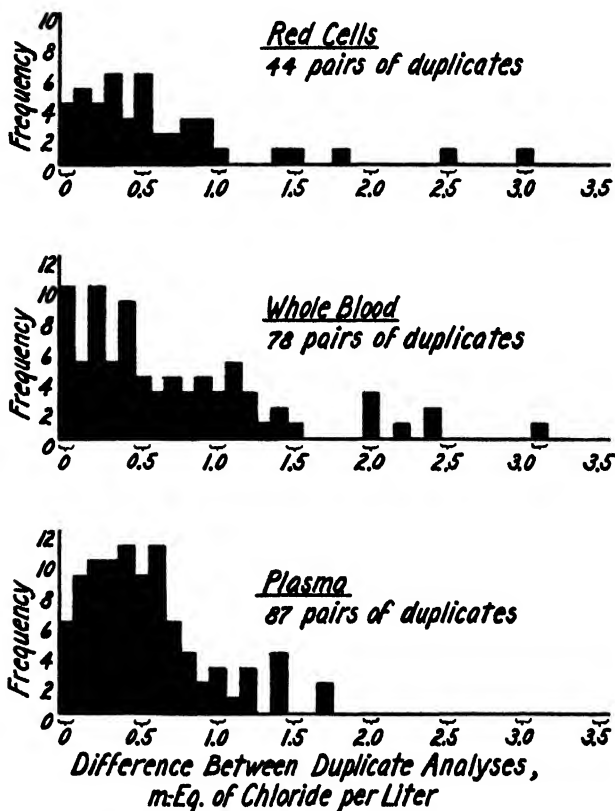


FIG. 3. Routine results from 2 months use of the present chloride method

1. In this laboratory duplicate analyses are the rule. In Fig. 3 I have summarized, in the form of frequency distribution charts, all the differences between duplicates covering a period of 2 months routine use of the present method (418 analyses). It is clear that gross discrepancies do not occur and that variations of as much as 1 milli-equivalent are infrequent. As would be expected, the

differences are somewhat smaller with plasma than with either whole blood or red cells.

2. A different and perhaps more useful measure of variability is obtained by repeated analyses on the same material. Two long series of this kind (thirty-seven and forty-two analyses, respec-

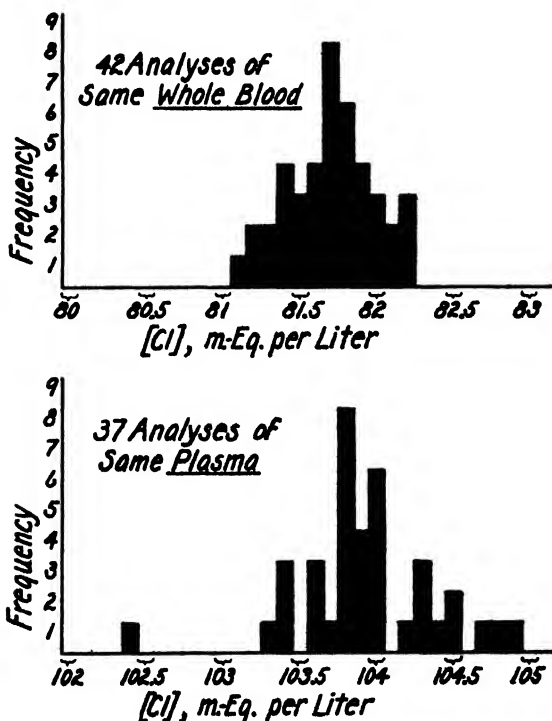


FIG. 4. Results of repeated analyses of the same material. Whole blood determinations were made by an experienced technician using more than routine care. Plasma determinations were made by an inexperienced technician following the instructions for the routine procedure.

tively) are summarized in Fig. 4. The standard deviation, in milli-equivalents per liter, for the plasma series analyzed by an inexperienced technician following a routine procedure is ± 0.465 ; in the second series (whole blood), run by an analyst experienced in the technique, $\sigma = \pm 0.282$.

3. The analysis of plasma to which have been added known

amounts of water or of chloride gives a reliable check on the accuracy of the method. The results of eight experiments of this kind are given in Table IV.

TABLE IV

Accuracy of Estimation of Chloride Added to or Subtracted from Blood Plasma Containing, Originally, 103 Milli-Equivalents of Cl per Liter

Mean values from duplicate analyses.

Water added				NaCl added			
Δ chloride		Error		Δ chloride		Error	
Found	Theoretical	Δ Cl	Total Cl	Found	Theoretical	Δ Cl	Total Cl
<i>m.-eq. per l.</i>	<i>m.-eq. per l.</i>	<i>per cent</i>	<i>per cent</i>	<i>m.-eq. per l.</i>	<i>m.-eq. per l.</i>	<i>per cent</i>	<i>per cent</i>
-29.7	-30.2	-1.65	-0.68	+20.9	+21.0	-0.48	-0.08
-42.6	-42.0	+1.43	+0.98	+42.6	+42.0	+1.43	+0.41
-48.4	-48.2	+0.41	+0.36	+52.2	+53.0	-1.51	-0.51
-69.8	-69.9	-0.14	-0.30	+70.5	+70.9	-0.56	-0.23
Mean.....		+0.01	+0.09			-0.28	-0.10

TABLE V

Comparison of Whole Blood [Cl] by Direct Analysis with [Cl] from Calculation from Separate Analyses of Plasma and Cells and the Measured Cell Volume (by Hematocrit)

Mean values, from duplicate analyses, given in milli-equivalents of [Cl] per liter. The blood was taken from hospital patients.

Patient	Cell volume	Cells	Plasma	Whole blood		Δ, as per cent of direct determination
				Direct	Calculated	
	<i>per cent</i>					
Gi	40.6	51.7	102.4	82.3	81.8	-0.61
Yo	47.6	49.6	98.5	74.4	75.2	+1.07
He	44.4	52.5	101.2	79.0	79.6	+0.76
Wh	42.7	53.8	102.0	81.4	81.4	0
Ga	38.0	52.1	102.2	61.8	62.3	+0.81
Fu	49.5	52.0	102.8	78.1	77.6	-0.64
Mean.....				76.17	76.32	+0.20

4. We have made many comparisons of the present method with macro-Volhard methods such as those of Laudat (1917), Van Slyke (1923-24), Wilson and Ball (1928), and others. In some

cases we have removed the precipitate of AgCl by centrifugation or filtration. Space does not permit an analysis of the detailed results here; we have found no significant difference between the results with the present method and the most careful application of the macroanalytical procedures. Finally, we have twice checked the method against the classical gravimetric method of Carius, using the open digestion procedure. On both of these occasions whole blood was used and the mean agreement between the two methods was within 0.3 milli-equivalent of Cl per liter.

5. Comparison of whole blood $[\text{Cl}]$ from direct analysis with the value for $[\text{Cl}]$ obtained by calculation from separate analyses of cells and plasma makes a sensitive test of the accuracy of the method. Some care is needed, however, to get a sufficiently accurate hematocrit value. For this latter purpose we have used specially calibrated 30 ml. hematocrit tubes in a refrigerated centrifuge for 2 hours at 2700 R.P.M. The results of six experiments are summarized in Table V.

DISCUSSION

Bjerrum (1914) has discussed in detail the factors making for precision in titrimetric procedures. It is clear that theoretical considerations favor the use of small volumes of relatively concentrated solutions so long as these may be measured with sufficient accuracy (see also Rehberg (1925, 1926), Keys (1931)). The requirements for precise microvolumetric measurements are fulfilled by the syringe-pipette and the Rehberg microburette when used properly.

The syringe-pipette has an absolute error of about ± 0.0001 ml. or a routine error of perhaps ± 0.0002 ml., which is nearly independent of the total volume of delivery between 0.1 and 1.7 ml. For the highest precision, then, the larger the delivery the better the results. With the Rehberg burette the absolute accuracy of the volume measurement is about ± 0.0002 ml. This would give a limiting accuracy of about ± 0.2 per cent when titration is completed at around 0.1 ml. In a back titration, however, the percentage accuracy of the final answer will be determined by the proportion of the total unknown represented by the titration. As used here the Ag^+ excess titrated is of the order of a third of the total chloride, so the theoretical limiting accuracy becomes about ± 0.07 per cent.

From the simple errors of volume measurements we might expect the general order of the maximum error in the method to be given by the sum of the maximum errors of the measurement of the CNS^- (± 0.07 per cent), of the measurement of AgNO_3 (± 0.04 per cent), and of the measurement of the sample (± 0.08 per cent); *i.e.*, about 0.2 per cent. To this, however, must be added the error in the recognition of the end-point; with titration at 0.1 ml. this amounts to about ± 0.0002 ml. or one-third of 0.2 per cent or about 0.07 per cent. The total maximum error then should be less than ± 0.3 per cent. Actually the maximum error is several times this value, so that volumetric accuracy is not yet the limiting factor.

A more detailed analysis of mean errors leads to a similar conclusion; *i.e.*, the errors actually found are of the order of 5 to 10 times those which could be expected from the known errors. Further improvements in the accuracy of the method cannot be expected to result from improvements in (1) measurement of the sample, (2) measurement of the AgNO_3 , (3) the measurement of the CNS^- required to titrate, (4) the recognition of the end-point.

SUMMARY

A method for the microanalysis of biological materials is presented. The method is not inferior in speed or accuracy to the best macroprocedures.

The method is analyzed in detail with regard to absolute and relative accuracy and the following factors are studied: measurement of volumes of sample and reagents, digestion, effects of storage, effect of light, prevention of back reactions, the end-point. The limiting errors are discussed.

Most of the analyses reported here were made by the following technicians: W. C. Consolazio, Catherine Ryan, Charlotte Bradshaw, and Margaret Haney.

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THE CARBOHYDRATE METABOLISM OF BRAIN
IV. BRAIN GLYCOGEN, FREE SUGAR, AND LACTIC ACID AS
AFFECTED BY INSULIN IN NORMAL AND ADRENAL-
INACTIVATED CATS, AND BY EPINEPHRINE
IN NORMAL RABBITS*

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In a recent publication (Kerr and Ghantus, 1936) it was shown that insulin causes a decrease in the brain glycogen of dogs and rabbits. It is uncertain, however, whether this change is a direct effect of insulin or a secondary effect resulting from the hypoglycemia, since it has been shown that insulin hypoglycemia evokes a reflex discharge of epinephrine from the adrenal medulla (Cannon, McIver, and Bliss, 1924) and that epinephrine causes a marked decrease in muscle glycogen in rats (Cori and Cori, 1928, *a,b*), rabbits (Sahyun and Luck, 1929-30), and dogs (Major and Mann, 1932¹). Corkill (1930), observing a decrease in the muscle glycogen of rabbits after insulin, attributed this to an increased production of epinephrine stimulated by the hypoglycemia. This interpretation is also presented by Cori (1931). We have therefore attempted to determine whether or not the changes observed in brain after insulin are actually effects of epinephrine production. The evidence presented in this paper indicates that the lowering of brain glycogen by insulin cannot be attributed to a stimulation of epinephrine secretion, since the same change occurs in adrenal-inactivated cats as in normal animals, and since the administration of adrenalin itself does not produce this effect.

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¹ Previous work is reviewed by these authors.

Effect of Insulin on Normal and Adrenal-Inactivated Cats

The effect of insulin in doses of 2 to 15 units per kilo was observed on the brain glycogen, free sugar, and lactic acid of normal and adrenal-inactivated cats. Inactivation of the adrenal medulla was accomplished by complete removal of the right adrenal gland and denervation of the left adrenal gland by section of the left splanchnic nerves and removal of the upper abdominal sympathetic trunks. In addition, the hepatic nerves were severed. The effectiveness of this method for preventing the secretion of epinephrine and similar compounds from the adrenals and the sympathetic nervous system has been tested by Cannon, McIver, and Bliss (1924), who were unable to detect any epinephrine secretion during insulin hypoglycemia in this preparation, using the denervated heart as an indicator. A period of 9 to 26 days was allowed for recovery and return to preoperative weight before studying the effect of insulin. The brain composition of control groups of operated and unoperated animals with no insulin was also determined. Unless otherwise noted in the tables, all animals were fasted 18 to 24 hours before the experiments. Amytal was used for anesthesia, Major and Mann (1932) having shown that this is without effect on the glycogen content of muscles.

The sampling of the brain and the chemical determinations were carried out as previously described (Kerr and Ghantus, 1936).

Results—Adrenal inactivation followed by a recovery period of 2 weeks resulted in no change from the normal levels of brain glycogen (Table I). The values obtained for free sugar and lactic acid in the "normal" cat brain are considerably higher than those found in normal dogs and rabbits (Kerr and Ghantus, 1936). On the other hand, the values found in the brains of adrenal-inactivated cats (with the exception of the high free sugar in Experiment 182) fall within the range of the normal established for dogs and rabbits. The high blood sugar observed in all the "normal" animals suggests that a discharge of epinephrine may have occurred from the adrenal medulla, owing to excitement during anesthesia. The values found for the adrenal-inactivated cats (Table I) form the basis for comparison with the insulinized animals in Table III.

Insulin in doses of 2 to 15 units per kilo of body weight caused a

lowering of glycogen and free sugar in the brains of all but two of fourteen normal cats examined (Table II), one exception (Experiment 200) being a pregnant animal. Similar doses of insulin given to adrenal-inactivated cats produced the same changes as those observed in unoperated animals (Table III). Symptoms of hypoglycemia developed far more rapidly in the operated animals and the lowering of brain glycogen was more pronounced. During

TABLE I

Control Experiments; Glycogen, Free Sugar, and Lactic Acid in Cerebrum of Normal and Adrenal-Inactivated Cats

All animals were fasted 18 to 24 hours; amytal anesthesia (intraperitoneally).

Experiment No.	Sex	Remarks	Blood sugar	Liver glycogen	Mg. per 100 gm. cerebrum		
					Glycogen	Free sugar	Lactic acid
			mg. per 100 cc.	per cent			
224	♀	Normal cat	210	2.2	97	107	30
225	♀	" "	170	0.5	78	116	24
226	♂	" "	205	3.9	85	90	22
227	♀	" "	178	4.5	101	92	25
228	♀	" "	154	1.4	72	92	22
Average					87	99	25
181	♂	Adrenal-inactivated cat	118	2.8	85	53	12
182	♀	" " "	153	4.2	81	127	14
183	♀	" " "	200	4.0	91	60	20
184	♀	" " "	102	3.1	87	50	10
185	♂	" " "	90	4.6	95	57	8
Average					88	69	13

hypoglycemia the lactic acid level in the brains of operated and unoperated animals averaged 10.4 and 8.8 mg. respectively, as compared with an average of 13 mg. for the adrenal-inactivated cats untreated with insulin. A higher level (25 mg.) was found in the normal unoperated cats, which also had relatively high blood sugar levels. This, as suggested above, may have been caused by a discharge of epinephrine from the adrenals.

The values found for liver glycogen show that symptoms of

TABLE II
Effect of Insulin on Glycogen, Free Sugar, and Lactic Acid in Cerebrum of Normal Cats
 Insulin given intraperitoneally after 18 to 24 hours fasting; amytal anesthesia.

Experiment No.	Sex	Insulin		Remarks	Amytal given	Brain frozen	Blood sugar mg. per 100 cc.	Liver glycogen per cent	Mg. per 100 gm. cerebrum		
		Dose	Time						Glycogen	Free sugar	Lactic acid
152*	♀	10	8.25 a.m.	Depressed	11.25 a.m.	11.35 a.m.			61	17	14
153	♂	10	8.33 "	No symptoms	11.25 "	11.39 "			75	21	11
154	♀	10	12.17 p.m.								
		10	2.50 "	Prostrated; respiration rapid	3 p.m.	3.10 p.m.	52		47	15	16
155	♀	10	8.23 a.m.								
		5	9.23 "								
		10	11.25 "	Prostrated	11.27 a.m.	11.45 a.m.	19		35	10	9
156	♂	10	8.32 "								
		5	9.32 "								
			10.10 "	Prostrated. Muscular twitching	10.13 a.m.	10.25 "	6		47	10	7
157†	♀	15	9.12 "								
			11.40 "	Prostrated; respiration rapid; twitching	?	12.10 p.m.	8		27	8	7
158†	♂	15	8.35 "								
		12	m.	Prostrated; respiration rapid							
			12.30 p.m.	" "	1.10 p.m.	1.20 "	36		42	14	8

159	♂	15	8.38 a.m.	Convulsions	11.37 a.m.	11.49 a.m.	31		44	10	9
			11.20 "	"							
204	♂	10	11.35 "								
			10.10 "								
200*	♀	5	12.15 p.m.	No symptoms	12.15 p.m.	1.06 p.m.	39	1.4	57	18	12
			8.04 a.m.		{ 10.12 a.m.						
			10.12 "	No symptoms	{ 10.40 "						
203	♀	5	9.50 "								
			11.45 "	Prostrated; respiration rapid; twitching	11.50 "	12.15 p.m.	28	3.4	54	18	8
201	♀	2	8.11 "								
			10.05 "	Depressed	10.15 "	10.40 a.m.	23	3.2	52	18	10
202	♀	2	8.25 "								
			10.30 "	No symptoms	10.30 "	10.58 "	30	4.2	75	18	10
205	♀	2	11.15 "								
			12.55 p.m.	No symptoms	12.55 p.m.	1.15 p.m.	47	1.6	58	21	12

* Found to be pregnant.

† We fed 20 gm. of fish before insulin was given in order to catch the animal.

186	♂	14	5	8 08 a.m. 9 00 "	Prostrated; respiration rapid Convulsions; artificial respiration	9 32 a.m.	9 41 a.m.	30	5 3	31	23	11
187	♂	14	5	8 10 " 8 55 "	Respiration rapid; pros- trated Respiration slows; con- vulsions	9 32 "	9 50 "	28	5 4	40	15	8
188	♀	9	2	9 10 " 9 55 " 10 15 "	Prostrated Respiration slows; con- vulsions	10 15 "	10 30 "	18	3 6	35	13	7
189	♀	14	2	9 02 " 9 50 " 10 20 "	Depressed Prostrated; artificial respiration	10 20 "	10 45 "	16	3 9	30	14	8
190	♂	13	2	9 06 " 10 20 " 10 30 "	Respiration rapid " slows; con- vulsions	10 52 "	11 00 "	25	3 9	24	26	11

* On autopsy found to be pregnant; fetuses 2.5 cm. long.

† On autopsy found to be pregnant; near term.

hypoglycemia and convulsions occur while the liver still retains at least as much glycogen as in the normal fasting animals. In most of the adrenal-inactivated cats the quantity of glycogen found in the liver at the time of convulsions is greater than in the normal fasting animals.

A comparison of the effect of insulin on brain with that on muscle glycogen is rendered somewhat difficult because of the lack of uniformity in the results presented by previous investigators on muscle.² These have been reviewed critically by Cori (1931) and by Sahyun and Luck (1929-30). The work of Cori and Cori (1928, b) as well as that of others reviewed by Cori (1931) points to the conclusion that insulin stimulates the transfer of sugar to the muscles both for storage as glycogen and for oxidation at a rate greater than normal. However, the majority of workers using fasted rabbits found either no significant change or a decrease in muscle glycogen after insulin (Cori, 1925; Sahyun and Luck, 1929-30; Goldblatt, 1929, 1930; Corkill, 1930), and this decrease was interpreted both by Cori (1931) and by Corkill (1930) as an effect of epinephrine reflexly discharged from the adrenal medulla.³ The effect of insulin in brain is therefore similar to that in muscle in that glycogen is lowered in both tissues in fasting rabbits, but the effect on brain glycogen cannot be attributed to the indirect action of epinephrine, as it has been for muscle, since the effect is not altered by inactivation of the adrenal glands.

This observation does not necessarily indicate that the mechanisms in brain operate differently from those in muscle. Insulin is known to cause an increased rate of carbohydrate oxidation in muscle (Cori and Cori, 1928, b). Should this be found true also for brain tissue, the increased rate of utilization could result only in a

² It is to be expected that varying results should be obtained, depending on the nutritional condition of the experimental animal, the time intervening between administration of insulin and analysis of the tissue (Sahyun and Luck, 1929-30), and the degree and duration of hypoglycemia. Thus Corkill (1930) found that insulin produced an increase in the muscle glycogen of 24 hour fasted chickens and ferrets, no increase with mice unless given glucose with the insulin, but a slight irregular decrease with young fasting rabbits.

³ In view of our findings on brain it appears desirable to test this interpretation by studying the effect of insulin on the muscles of adrenal-inactivated animals.

depletion of brain glycogen, since after insulin the supply of blood sugar for glycogenesis in brain is diminished, owing to withdrawal by the muscles at a rate exceeding glycogenolysis in the liver. Although the liver retains a considerable supply of glycogen at the time of convulsions, this appears to be unavailable for other tissues, owing to inhibition of glycogenolysis by insulin (Cori, Cori, and Goltz, 1923; Goldblatt, 1929; Cori, 1931). We have previously shown that severe depletion of the carbohydrate reserves by phlorhizin (uncomplicated by the factor of increased rate of utilization (Deuel, Wilson, and Milhorat, 1927)) leads to no decrease of brain glycogen (Kerr and Ghantus, 1936). It appears therefore that the lowering of glycogen in brain by insulin must be the result of an effect on the brain tissue itself (presumably an increase in the rate of utilization of carbohydrate), combined with an interference with the supply necessary for replacement, but not to a withdrawal of carbohydrate from brain to muscle.

Examination of Tables II and III for evidence of a correlation between the chemical findings and the symptoms usually attributed to hypoglycemia reveals a striking difference between the adrenal-inactivated cats and those with an intact mechanism for secreting epinephrine. Of the insulinized unoperated cats, definite convulsions occurred in only one (Experiment 159) out of fourteen, whereas seven of the ten adrenal-inactivated cats went into convulsions and three passed into a state of coma with respiratory failure without convulsions. In the normal (insulinized) cats the state of depression is accompanied by glycogen levels approximately within the range 47 to 56 mg. per 100 gm. of brain. Glycogen levels below 47 mg. are accompanied by inability to stand, complete prostration, muscle twitchings, or convulsions. In cats deprived of the mechanism for secreting epinephrine, convulsions or coma occurs regularly at levels below 50 mg. There appears to be no correlation between the level of free sugar and the symptoms which follow insulin. Similar experiments on normal dogs (Kerr and Ghantus, 1936) did not show the same results.⁴

⁴ The injection of amytal stops the convulsions promptly, and during the period of 10 minutes or more which elapses before the brain can be frozen recovery mechanisms may restore the brain to a more normal chemical composition. It is desirable to reexamine the question of a possible correlation between hypoglycemic symptoms and chemical findings, with an anesthetic which will permit more prompt fixation of the brain.

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If our hypothesis that insulin lowers brain glycogen by increasing the rate of utilization of carbohydrate in this tissue is correct, the greater sensitivity to insulin noted in the adrenal-inactivated animals would appear to be due to removal of a mechanism which after insulin slows down the utilization of carbohydrate in the tissues.

Effect of Epinephrine on Normal Rabbits

Since it has been shown that epinephrine causes a decrease in the glycogen content of skeletal muscle (Cori and Cori, 1928, *a, b*; Sahyun and Luck, 1929-30; Cori, 1930; Corkill and Marks, 1930; Major and Mann, 1932¹), it remains to be shown whether or not direct administration of epinephrine has a similar effect on the glycogen of brain.

Rabbits of about 2 kilos weight, previously fed on barley and alfalfa, were used for these experiments. After 24 hours fasting⁵ epinephrine⁶ was administered by one of the methods described in more detail below, and at the end of the experimental period the animals were anesthetized with amytal. The muscle sample (a portion of the quadriceps femoris) was next excised and plunged into liquid air, after which the brain was frozen *in situ* with skull intact. Finally a piece of liver was rapidly removed and frozen, and blood was aspirated from the heart, which in all cases was still beating. During these operations respiration was maintained by artificial means, since natural breathing ceases 1 to 2 minutes after starting to freeze the brain.

The analysis of muscle, liver, and brain samples was carried out according to the methods previously described for brain (Kerr and Ghantus, 1936).

Normal fasting rabbits with no epinephrine injections were also studied. The results of these control experiments are presented in Table IV. The values for muscle glycogen in the control group (0.42 to 0.91 per cent, average 0.60) are similar to those obtained by Sahyun and Luck (1929-30) (0.40 to 0.62 per cent, average

⁵ In Experiments 196 and 197 the period of fasting was unknown. In Experiment 113 the period was only 5 hours (see Table V).

⁶ Adrenalin chloride of Parke, Davis and Company was used in all experiments.

0.50).⁷ Brain glycogen, free sugar, and lactic acid values are also similar to those previously reported (Kerr and Ghantus, 1936).

As a preliminary study we administered epinephrine in a variety of ways and doses, and studied the effect on brain composition after intervals of 18 to 78 minutes. No effect was observed on brain glycogen, either with single doses of 0.2 mg. per kilo given by subcutaneous, intraperitoneal, or intravenous injection or with two such doses at intervals (Table V). Although the dosage of epinephrine in all of these experiments was sufficient to produce

TABLE IV

Glycogen, Free Sugar, and Lactic Acid in Cerebrum of Normal Fasting Rabbits; Amytal Anesthesia

Rabbits fasted 24 hours except in Experiment 210, fasted 28 hours.

Experiment No.	Sex	Blood sugar	Liver glycogen	Muscle glycogen	Mg. per 100 gm. cerebrum		
					Glycogen	Free sugar	Lactic acid
		mg. per 100 cc.	per cent	per cent			
209	♂	121		0.91	120	65	16
210	♀	82		0.42	91	43	22
211	♀	110	0.67	0.80	68	58	26
212	♀	105	1.23	0.74	84	70	13
218	♂	85	0.26	0.53	70	75	21
219	♀	110	1.12	0.43	85	66	12
220	♂	107	0.94	0.47	93	51	15
Average		103	0.84	0.60	87	61	18

hyperglycemia and a raised level of free sugar in the brain, the lactic acid of the brain was not significantly altered.

We next followed the experimental procedure which, according to Sahyun and Luck (1929-30), produces maximum changes in muscle glycogen after epinephrine. They observed that a subcutaneous injection of 1 mg. of epinephrine produced in rabbits fasted for 24 hours a sharp lowering of muscle glycogen which

⁷ We determined the lactic acid content of muscle in a number of these animals in order to learn what amount of glycogenolysis had taken place. The values obtained varied from 38 to 61 mg. per cent (average 53), showing that although "resting" values had not been secured, the error introduced was not sufficient to vitiate our conclusions.

TABLE V
Effect of Epinephrine on the Glycogen, Free Sugar, and Lactic Acid in Cerebrum of Normal Rabbits

Dosages of amytal and epinephrine are in terms of mg. per kilo of body weight; amytal by intravenous injection, unless otherwise noted.

Experiment No.	Sex	Period of fasting	Time	Interval between epinephrine and freezing brain	Remarks	Blood sugar mg. per 100 cc.	Liver glycogen per cent	Mg. per 100 gm. cerebrum		
								Glycogen	Free sugar	Lactic acid
195	♀	27	10.34 a.m.	18	Amytal, 100 mg.	180	0.23	92	108	28
			10.40 "		Adrenalin, intravenous, 0.2 mg.					
			11.58 "		Brain frozen					
196	♀	?	9.00 "	22	Amytal, 90 mg.	182	1.12	99	110	16
			9.11 "		Adrenalin, subcutaneous, 0.2 mg.					
			9.33 "		Brain frozen					
197	♂	?	10.17 "	23	Adrenalin, subcutaneous, 0.2 mg.	170	1.34	105	91	13
			10.27 "		Amytal, 80 mg.					
			10.40 "		Brain frozen					
113		5	3.15 p.m.	28	Amytal, intraperitoneal, 80 mg.	200	2.46	82	101	12
			3.16 "		Adrenalin, subcutaneous, 0.2 mg.					
			3.36 "		Amytal, 30 mg.					
193	♀	26	3.44 "	45	Brain frozen	245	1.16	107	120	15
			9.15 a.m.		Adrenalin, intraperitoneal, 0.2 mg.					
			9.32 "		Amytal, intraperitoneal, 90 mg.					
			9.42 "		Adrenalin, subcutaneous, 0.2 mg.					
			9.46 "		Amytal, 20 mg.					
			10.00 "		Brain frozen					

194	♀	26	9.48 a.m. 9.50 " 10.10 " 10.50 " 11.08 "	78	Amytal, intraperitoneal, 100 mg. Adrenalin, subcutaneous, 0.2 mg. Amytal, intravenous, 83 mg. Adrenalin, intravenous, 0.2 mg. Brain frozen	270	0.24	111	167	15
Average.	208	1.09	99	116	17

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persisted from 1½ to 42 hours. On the assumption that this dosage represents approximately 0.5 mg. per kilo, we injected this amount, and sampled muscle, liver, and brain after an interval of 2 to 3 hours. This procedure resulted in a marked hyperglycemia,

TABLE VI

Effect of Epinephrine (0.5 Mg. per Kilo) on Glycogen, Free Sugar, and Lactic Acid of Brain, and Glycogen of Liver and Muscle, in 24 Hour Fasted Rabbits

Adrenalin given subcutaneously; amytal intravenously.

Experiment No.	Sex	Time	Interval between epinephrine and freezing brain	Remarks	Blood sugar	Liver glycogen	Muscle glycogen	Mg. per 100 gm. cerebrum		
								Glycogen	Free sugar	Lactic acid
			min.		mg. per 100 cc.	per cent	per cent			
214	♀	12 m. 2.15 p.m. 2.27 "	147	Adrenalin Amytal Brain frozen	392	0.10	0.097	95	187	22
215	♀	11.30 a.m. 1.15 p.m. 1.44 "		Adrenalin Amytal Brain frozen						
216	♀	11.10 a.m. 1.55 p.m. 2.16 "	186	Adrenalin Amytal Brain frozen	366	0.66	0.088	82	157	21
221	♀	9.00 a.m. 11.10 " 11.26 "		Adrenalin Amytal Brain frozen						
222	♂	9.50 " 11.35 " 11.48 "	118	Adrenalin Amytal Brain frozen	289	0.28	0.111	87	123	17
223	♀	10.10 " 11.55 " 12.09 p.m.		Adrenalin Amytal Brain frozen						
Average.....					349	0.36	0.113	89	155	21

with a corresponding but smaller rise in the free sugar of brain, a sharp decrease in muscle glycogen, but no effect on the glycogen or lactic acid content of brain (Table VI).

The liver appears to suffer a loss of glycogen during the 2 or 3

hour period following the subcutaneous injection of 0.5 mg. of epinephrine per kilo, since the normal livers have values between 0.26 and 1.23 per cent (average 0.84), while the livers of the injected animals contain 0.10 to 0.66 per cent (average 0.36) (Table VI). In two of the control animals, however, the liver glycogen values fall within the range of those injected with epinephrine. These results confirm the findings of Sahyun and Luck (1929-30), who noted a fall in liver glycogen during the first 2 hours after

TABLE VII

Effect of Repeated Dosage with Epinephrine on Glycogen, Free Sugar, and Lactic Acid of Rabbit Brain

Epinephrine administered intraperitoneally; amytal intravenously.

Experiment No.	Sex	Period of fasting hrs.	Epinephrine dosage per in- jection mg. per kg.	Interval between injections min.	No. of injections	Interval be- tween in- jection and fasting brain min.	Blood sugar mg. per 100 cc.	Liver glycogen per cent	Mg. per 100 gm. cerebrum		
									Glycogen	Free sugar	Lactic acid
206	♂	20	0.05	20	7	120	140	8.91	69	98	19
207		20	0.05	20	7	120	373	6.92	76	172	19
208		20	0.05	20	7	120	288	5.32	73	124	25
232	♀	24	0.5	30	4	115	624	0.51	110	186	30
235	♀	24	0.5	30	4	120	236	0.03	83	141	19
233	♂	24	0.5	30	4	165	307	0.06	108	149	17
234	♂	24	0.5	30	6	210	350	0.11	107	177	15
236	♂	24	0.5	30	8	240	380	0.08	87	194	16
237	♂	24	0.5	30	8	255	307	0.05	95	182	11

epinephrine, after which a rise above the normal fasting level occurred.

Repeated doses of 0.05 mg. of adrenalin per kilo (intraperitoneally) at 20 minute intervals over a period of 2 hours produced brain glycogen values around the lower limits of the normal range (Experiments 206, 207, 208, Table VII), but the change was in no way comparable to that caused by insulin hypoglycemia (Kerr and Ghantus, 1936). A large increase in the glycogen content of liver took place as a result of the epinephrine injections.

Finally we studied the effects of the massive doses used by Nahum and Himwich (1931) in their experiments on the lactic acid

exchange between blood and brain. Epinephrine was administered at half hour intervals in doses of 0.5 mg. per kilo of body weight.⁸ Brain glycogen and lactic acid remained within normal limits following four, six, or eight such doses (Experiments 232 to 237, Table VII). The free sugar of the brain rose in response to the hyperglycemia. Liver glycogen decreased to very low levels.

Our findings in regard to the lowering of muscle glycogen by epinephrine confirm those of Sahyun and Luck (1929-30), Major and Mann (1932),¹ Bridge and Noltie (1935), and others. With a dosage of epinephrine sufficient to produce a marked lowering of glycogen in skeletal muscle, the brains of the same animals suffer no detectable loss of glycogen, and in this respect resemble heart muscle (Long and Evans, 1932-33).

Cori and Cori (1930) showed that the rate of absorption of epinephrine from the subcutaneous tissue after injection of 0.2 mg. per kilo is, at least for the rat, within the limits of the physiological output of the adrenals as determined by Cannon and Rapport (1921). Since neither this dosage nor 0.5 mg. per kilo produced any change in brain glycogen, we may conclude that epinephrine in doses within the physiological range causes no significant glycogenolysis in brain.

Nahum and Himwich (1931) found that, although small doses of adrenalin (0.5 cc. per $\frac{1}{4}$ hour per dog) did not raise the lactic acid content of the blood from the superior longitudinal sinus above that of the femoral arterial blood, massive doses (5 cc. per half hour) increased the lactic acid in the cerebral blood above that in the femoral artery within $1\frac{1}{2}$ hours. This indicates that the brain liberated lactic acid under the influence of two such doses. Our experimental results indicate that no significant glycogenolysis occurs under these conditions. Moreover, if lactic acid is produced from some other source, it is disposed of with such efficiency that the level in the brain does not rise significantly above normal.

SUMMARY

The effect of insulin in doses of 2 to 15 units per kilo was determined on the brain glycogen, free sugar, and lactic acid in normal and in adrenal-inactivated cats.

⁸ Nahum and Himwich injected 5 cc. of adrenalin per half hour, but did not state the weight of the dogs or the mode of administration.

Insulin decreases the level of glycogen and free sugar in the brain of normal cats and to an even greater degree in the animals made incapable of secreting epinephrine.

It is concluded that the effect of insulin on brain glycogen cannot be attributed to the indirect action of secreted epinephrine, since the lowering of glycogen is not abolished by inactivation of the adrenal medulla.

Glycogen levels below 50 mg. per 100 gm. are accompanied by prostration and incoordination in cats with intact adrenals, but with convulsions in adrenal-inactivated cats.

Epinephrine in doses sufficient to produce marked losses of glycogen from liver and skeletal muscle does not significantly change the glycogen and lactic acid content of the brain in fasting rabbits.

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THE DISTRIBUTION OF FAT IN THE LIVERS OF DEPANCREATIZED DOGS MAINTAINED WITH INSULIN*

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Although it has been the practise in this laboratory to employ mixed samples of the entire liver in order to estimate the lipid or fatty acid content of this organ (1), other workers have resorted to removal of small sections or of an entire lobe for this determination. The method by which a few gm. of the liver are removed for fat determinations has undoubted advantages in the depancreatized dog, for it provides a means for comparing the amount of fat deposited at various intervals in the liver of the same animal. An even distribution has apparently been assumed in the fatty livers of such animals, although no satisfactory evidence has been provided to establish this belief in respect to the completely depancreatized dog, an animal in which various degrees of lipid infiltration may occur.

In view of the many studies being made on the liver lipids of the depancreatized dog, the question of the validity of employing a few gm. of a liver for determination of its fat content is obviously of importance, since the use of a mixed sample of the whole liver involves sacrificing the animal. It is shown in the present investigation that the deposition of fat does not necessarily take place in a uniform manner in the completely depancreatized dog maintained with insulin. In a number of cases the fat content of one section varied considerably from that of another section or from that of a mixed sample of the entire liver.

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Since normal as well as fatty portions may exist side by side in the same liver, it is obvious that the lipid content of a single section may be entirely misleading in regard to the lipid content of the entire liver.

EXPERIMENTAL

Female dogs were used throughout this investigation. Following complete pancreatectomy, the animals received twice daily, at 8 a.m. and 4 p.m., a diet mixture of lean meat, sucrose, bone ash, vitamins, and, from time to time, other supplements. The animals were injected with 8 units of insulin at each time of feeding, and finally were sacrificed while in good nutritional condition at periods varying from 3.5 to 36.5 weeks after pancreatectomy.

Sampling of Liver—Livers were excised between 8 and 10 a.m. under anesthesia induced by sodium amytal. Unless otherwise specified, the animals received their last meal and last injection of insulin at 4 p.m. of the previous day. The surface of the liver was wiped clean of blood and separated into individual lobes, which are classified according to Ellenberger and Baum (2).

Three types of sampling were employed in the various livers: (1) whole lobes; each lobe was cut into small pieces with scissors and placed directly in a weighed flask containing 95 per cent ethyl alcohol.¹ (2) One or more small sections were removed from each lobe; the rest of the liver was thoroughly ground and mixed. A portion of the mixed sample was then taken for analysis. The small sections measured approximately $15 \times 15 \times 2$ mm. and were removed from the center or near the periphery of a lobe. Immediately after removal each section was placed in a weighed vial containing 95 per cent alcohol. In the case of the mixed sample, 15 gm. portions were employed for analyses. (3) A small section of the liver was removed from each lobe, while the rest of the lobe was cut into small pieces and analyzed for its lipid content.

¹ All solvents employed in this investigation were purified and freshly distilled before use. All apparatus, including joints, used in the analytical procedures and in the purification of solvents were constructed entirely of glass. All extracts were made up to volume at 20° and brought back to this temperature before aliquot portions were removed, since the solvents employed have relatively high coefficients of expansion.

Determinations of Total Fatty Acid

Small Sections—Each section of liver was transferred to a mortar and thoroughly ground with a small amount of purified sand which had been extracted with alcohol and ether. The ground material, along with the sand and the alcohol in which the tissue had been stored, was transferred quantitatively with alcohol to a 125 cc. Erlenmeyer flask. The hashed liver was extracted at 55–60° for 1.5 to 2 hours with 50 cc. of alcohol. The alcohol was then decanted through a fat-free filter paper into a 200 cc. volumetric flask. The extraction of the tissue was repeated with a fresh 50 cc. portion of alcohol for 1.5 to 2 hours. The extract was filtered into the volumetric flask previously used and the tissue residue quantitatively transferred to the filter paper. The residue, wrapped in its filter paper, was placed in a Soxhlet apparatus and continuously extracted with ethyl ether for a period of 8 to 12 hours. The ether extract was then transferred to the volumetric flask containing the combined alcohol extracts, and the mixture made up to volume with ethyl ether.

Total fatty acids were determined after the manner previously described for blood (3). Aliquot portions of the alcohol-ether extract were saponified with sodium ethylate; following acidification, the fatty acids therein were extracted with petroleum ether and then made up to volume. Aliquot portions of the petroleum ether extract were removed for the determination of total fatty acids by the microoxidative method of Bloor (4), corrections being made for total cholesterol which was determined by the digitonide method of Okey (5) as modified in this laboratory (3).

Mixed Sample—Total fatty acids in the 15 gm. portions of the mixed sample of the entire liver were determined by the macro-method previously described (1).

In those cases in which each whole lobe was analyzed separately the fatty acid content of a mixed sample was obtained from the following ratio

$$\frac{W_1 F_1 + W_2 F_2 + \dots + W_6 F_6}{W_1 + W_2 + \dots + W_6}$$

where W and F represent, in the six lobes comprising the liver, the weight of each lobe and its percentage of fatty acids respectively.

TABLE I
Distribution of Total Fatty Acids in Liver As Shown by Analyses of Whole Lobes and Sections of Lobes

The values are expressed as per cent of fresh tissue. Values for the fatty acids in separate sections of a lobe together with the weight of the corresponding sections are set in bold-faced type.

Dog No.	Portion of lobe	Lobes of liver												Fatty acids in mixed sample of entire liver
		Left main lobe		Right middle lobe		Left middle lobe		Right main lobe		Lobus spigelii		Lobus quadratus		
										Weight of sample	Fatty acids	Weight of sample	Fatty acids	
		gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent					
D-103	Sections	1.313	10.0	0.491	18.5	0.787	17.3	0.610	28.4	0.622	27.6			
	Whole	79.55	18.6	0.752	13.4	45.40	16.5	75.10	27.0	37.35	29.6	15.62	21.8	
	Sections	0.718	7.09	0.473	11.4	0.358	7.30	0.660	6.15	0.398	10.0	0.508	6.60	
D-91	Whole	0.533	6.61					0.470	11.5					
	Whole	110.90	9.06	93.65	8.90	42.30	6.63	64.10	8.00	40.40	9.14	15.48	10.9	
	Sections	0.839	4.68	0.949	7.54	1.038	7.53	1.142	6.40	0.675	4.00	0.574	8.23	
D-105	Whole	1.148	7.94											
	Whole	0.921	7.09											
	Sections	166.20	6.79	90.10	7.50	95.45	7.11	84.55	6.20	63.35	7.14	26.98	7.10	
D-99	Sections	0.933	5.41	0.686	3.85	0.441	5.21	0.471	5.84	0.641	6.93	0.273	6.59	
	Whole					0.503	4.29			0.425	8.53			
	Whole	74.00	6.73	42.70	4.20	29.70	4.96	70.20	5.90	38.35	7.74	16.31	5.07	
D-102	Sections	0.589	4.47	0.473	5.64	0.492	5.23	0.746	5.40	0.574	4.82	0.527	5.53	
	Whole			0.321	4.24			0.563	3.98					
	Whole	87.00	4.51	80.40	4.60	45.50	4.71	70.30	4.56	26.20	4.47	21.61	4.62	
													4.58*	

D-85	Sections	0.709	26.9	0.658	22.7	0.917	30.0	0.416	28.0	0.510	19.1	0.512	27.0
	Whole			0.709	22.2					0.510	25.3		
D-106	"	156.60	26.7	109.20	28.0	77.85	29.0	118.10	27.9	58.80	23.7	18.42	25.9
D-82	"	66.80	33.1	37.15	32.7	46.60	33.1	37.35	30.8	27.55	29.2	7.38	34.8
D-77	"	111.15	12.0	62.75	9.97	85.40	10.0	67.64	11.6	49.60	13.1	19.47	11.6
D-81	"	83.90	5.70	78.50	5.77	85.00	4.88	62.90	5.32	29.57	3.94	18.03	6.66
D-100	Sections	1.713	16.2	1.706	13.1	1.784	15.0	2.039	9.28	1.220	10.2	1.100	15.1
	"	3.248	10.5	2.351	10.4	2.136	11.1	1.571	13.5	2.044	12.2	2.063	10.4
D-85†	"	3.255	10.6					2.301	12.5	3.407	11.4		
D-73	"	2.394	48.5	1.458	50.2	2.220	48.9	2.375	52.5	1.851	49.4	1.937	50.4
	"	2.719	22.9	3.072	21.8	3.650	22.6	4.600	14.4	1.134	18.8		
D-70	"	2.063	26.8										
	"	1.061	5.14	1.384	4.41	2.153	5.16	1.843	4.54	0.587	7.30	1.635	5.73
D-94	"	3.273	7.57	0.734	5.04	1.278	4.29	1.987	17.5	1.577	19.7	1.406	8.71

* Calculated from the weights and percentages of fatty acids in the whole lobes.

† Determined by analyses of mixed samples of the entire liver.

‡ Received no insulin for 6 days prior to removal of the liver.

Whole Lobes—Total fatty acids of whole lobes were determined in a manner similar to that for the mixed sample, but modifications were introduced to expedite the treatment of the larger quantities of tissue. Whole lobes were saponified for 4 hours on a steam bath with 100 to 250 cc. of 10 to 15 per cent solution of potassium hydroxide in 95 per cent alcohol. On cooling, the hydrolyzed mixture was transferred quantitatively with water to volumetric flasks and made up to 500 cc. volume. Suitable aliquots were transferred to separatory funnels and sufficient water was added to reduce the concentration of alcohol to 25 per cent. The extraction and determination of total fatty acids henceforth were the same as described above for the mixed sample.

All determinations were carried out in duplicate or triplicate, and the values recorded are the averages of closely agreeing results.

Results

The livers of fifteen completely depancreatized dogs were examined at intervals of 3.5 to 36.5 weeks after pancreatectomy. Their fatty acid content varied from 4.6 to 49.2 per cent (mixed sample). In each case the fatty acid content of the whole liver was determined, and this was compared with the fatty acid content found in whole lobes and in small sections removed from each lobe. In several animals, two or more small sections of liver were removed from the same lobe and their lipid content compared. This treatment permitted the following comparisons to be made.

Fatty Acid Content of Various Whole Lobes of Same Liver—Each of the six lobes of nine livers was analyzed for its total fatty acid content and the values obtained are recorded in Table I. In one of these, namely Dog D-102, the lipid content of the various lobes was in very close agreement. The maximum and minimum values were 4.7 and 4.5 per cent. In Dog D-99, however, a fluctuation between 4.2 and 7.7 per cent was observed. A similar variation was also found in Dog D-77, in which the percentage of fatty acid varied between 3.9 and 6.7 per cent. Three livers examined had a fat content above 20 per cent. In two of these (Dogs D-85 and D-106) an absolute difference of approximately 5 per cent was observed among the various lobes. In Dog D-103, on the other hand, a most remarkable difference

among the various lobes was noted. The highest and lowest values were 16.5 and 29.6 per cent respectively (Table II).

Distribution of Fatty Acids within Single Lobe—To ascertain the uniformity of distribution of fatty acids within a single lobe, two or three small portions were removed from each of fifteen

TABLE II
Degree of Variation in Fatty Acid Distribution in Liver

Dog No.	Portion of lobes	Range of values	Absolute difference between maximum and minimum values	Deviation from value for mixed sample	
				In case of minimum value	In case of maximum value
		<i>per cent of fresh tissue</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
D-103	Sections	10.0 -28.4	18.4	53.7	31.5
	Whole	16.5 -29.6	13.1	23.6	37.0
D-91	Sections	6.15-11.5*	5.35	28.9	33.0
	Whole	6.63-10.9	4.27	23.4	26.0
D-105	Sections	4.00- 8.23	4.23	42.5	16.3
	Whole	6.20- 7.50	1.30	12.4	5.9
D-99	Sections	3.85- 8.53	4.68	36.3	41.2
	Whole	4.20- 7.74	3.54	30.5	28.2
D-102	Sections	3.98- 5.64	1.66	13.1	23.2
	Whole	4.47- 4.71	0.24	2.4	2.8
D-85	Sections	19.1 -32.2	13.1	29.8	18.4
	Whole	23.7 -29.0	5.3	12.9	6.6
D-106	"	29.2 -34.8	5.6	8.2	9.4
D-82	"	9.97-13.1	3.13	10.3	18.0
D-77	"	3.94- 6.66	2.72	26.4	24.5
D-81	Sections	9.28-16.2	6.92	30.2	21.8
D-100	"	10.4 -13.5	3.1	5.5	22.7
D-95	"	48.5 -52.5	4.0	1.4	6.7
D-73	"	14.4 -26.8	12.4	28.0	34.0
D-70	"	4.41- 7.30	2.89	23.2	27.2
D-94	"	4.29-19.7	15.4	59.5	85.8

* Both samples occurred in the same lobe.

lobes. The values obtained for fatty acids, along with the weights of the sections removed for analysis, are shown by the bold-faced figures of Table I. In a number of cases, a sensible agreement in the fatty acid content of the two sections of the same lobe was observed. But in others divergences were found. The

left main lobe of Dog D-105 weighed 166 gm., and from this three samples of approximately 1 gm. each were removed for lipid analysis. Two of these contained 7.9 and 7.1 per cent, while the third sample contained 4.7 per cent. Significant, however, is the result obtained with Dog D-85, in which two samples, removed from the right middle lobe, showed a fatty acid content of 23 and 32 per cent. Dog D-91 provided an interesting result in that, whereas the two sections removed from the left main lobe contained approximately the same amount of fatty acids, namely 6.6 and 7.1 per cent, the two removed from the right main lobe contained 6.2 and 11.5 per cent.

Comparison between Fatty Acid Content of Whole Lobe and of Sections Derived from Same Lobe—In six livers the lipid content of whole lobes was compared with small portions, usually 1 gm. or less, removed from these lobes. In three lobes noteworthy differences were observed. In the first animal recorded in Table I (Dog D-103), the whole of the left main lobe weighed 79.6 gm., and a 1.3 gm. portion was removed from it. The fatty acid content of the small section was 10.0 per cent, whereas the whole lobe contained 18.6 per cent. In Dog D-105 the fatty acid content of the whole lobus spigelii and of a section removed from it was respectively 7.1 and 4.0 per cent. In Dog D-91 the fatty acid content of the whole quadrate lobe and of a section (0.5 gm.) removed from it was respectively 10.9 and 6.6 per cent. Although in a number of other cases discrepancies between a section and its whole lobe were found, these were not so great as in the three recorded above.

Comparative Lipid Content Among Single Sections Removed from Each of Six Lobes of Same Liver—Since biopsy samples have been used to compare, from time to time, the degree of fat infiltration in the liver of the same animal, it is obviously necessary to determine whether sections removed at random from each of the lobes of the same liver contain similar amounts of fat. Tables I and II show quite clearly that the fatty acid content of a small portion of one lobe need not reflect the content of a section of another lobe. Portions weighing 0.5 to 1.3 gm. were removed from each of the lobes of Dog D-103, and in these were found fatty acids varying from 10.0 to 28.4 per cent. The most striking fluctuations occurred in Dog D-94, in which sections removed

from each lobe fluctuated between 4.3 and 19.7 per cent. Table II shows the maximum and minimum values observed in twelve livers. In only three or four of these were the values for the various sections removed from the lobes of a single liver in close agreement. These results leave little doubt that the removal of small portions of liver (in the neighborhood of 0.5 to 3 gm.) cannot be employed for an accurate index of the lipid infiltration in the depancreatized animal. When such differences as shown by Dog D-94 can occur, it is apparent that the lipid content of one section of liver may be entirely misleading in regard to the fat content of another section.

Fatty Acid Content of Section or of Entire Lobe Compared with Lipid Content of Mixed Sample of the Whole Liver—The final question that remains to be considered is whether the lipid content of small sections or of a whole lobe is indicative of the lipid content of the entire liver. The fatty acid content of mixed samples of fifteen whole livers is shown in Table I. This was determined by analyses either of mixed samples of the whole liver or calculated from values obtained from the analyses of individual lobes. Table I shows quite clearly that the fatty acid content of a small section of the liver does not necessarily represent the lipid content of the whole liver. The mixed sample of the whole liver of Dog D-103 contained 21.6 per cent fatty acids, while a section removed from the left main lobe contained only 10.0 per cent. Sections removed from the left middle lobe and the lobus spigelii of Dog D-94 contained 4.3 and 19.7 per cent respectively, whereas the mixed sample of the whole lobe contained 10.6 per cent.

The use of whole lobes would seem to be more satisfactory as an index of the fatty acid content of the whole liver, but even so a close correlation between the lipid content of whole lobes and that of the entire liver is not always obtained (cf. Dogs D-103 and D-91).

DISCUSSION

The results of the present investigation show quite clearly that when the lipid content of the liver is increased in the depancreatized dog, fatty acids are not deposited uniformly throughout the organ. The extent of the variation is summarized in Table II.

The liver of Dog D-94 in particular is worthy of note, since sections containing normal as well as fatty portions were removed. In four livers absolute differences of 12 to 19 per cent were found between maximum and minimum values. The per cent deviations in value between individual slices and the mixed sample may become as great as 86 per cent.

When the lipid infiltration approaches saturation, fatty acids are probably uniformly distributed throughout the liver. This is borne out by the liver of Dog D-95, in which fatty acids were present to the extent of 49.2 per cent (mixed sample). Similarly, a somewhat uniform distribution is to be expected in livers in which the mixed sample shows a normal or low lipid content. Although the percentage deviations of the various samples may appear large in such livers (Dog D-102), nevertheless the absolute difference to be found among the various sections is small.

Whole lobes provide a more satisfactory basis for comparison of the fatty acid content of the livers of depancreatized dogs, but even here one of the nine livers examined showed an absolute difference of 13 per cent in the values obtained for the six lobes (Dog D-103). The deviation of individual lobes from a mixed sample may be as great as 37 per cent.

Dragstedt *et al.* (6) have recently reported the isolation of a fraction from the pancreas capable of curing fatty livers of depancreatized dogs. For assay they have employed depancreatized dogs in which the fat content of biopsy samples of the liver was compared in the same dog by histological means before and after the feeding of pancreatic fractions. While fatty acids may be evenly distributed in the liver of some depancreatized dogs, in others the variation among different parts may be as great as subsequent changes induced by an extract. Since there is no way of predicting in which liver and at what period after pancreatectomy an even distribution of fatty acids will be present, it is highly doubtful whether small portions of the liver provide the most satisfactory method for comparing the fatty change in the liver from time to time. Since fatty as well as normal portions (19.7 and 4.3 per cent total fatty acids respectively) may exist simultaneously in the same liver, the validity of conclusions drawn from comparisons of the lipid content of small liver sections in depancreatized dogs may indeed be questioned.

SUMMARY

1. In fifteen completely depancreatized dogs, a comparison was made between the fatty acid content of a mixed sample of an entire liver and the amount of fatty acids contained in its lobes and sections of these lobes. The values obtained for these three types of sampling show that fatty acids are not evenly deposited in the liver as fat accumulates.

2. The validity of employing whole lobes and sections of lobes to compare the fatty acid content of the whole liver at various intervals after pancreatectomy is discussed.

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THE EFFECT OF RAW AND AUTOCLAVED PANCREAS ON THE LIVER LIPIDS OF THE COMPLETELY DEPANCREATIZED DOG MAINTAINED WITH INSULIN*

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The presence of a substance in raw pancreas capable of influencing the level of the blood lipids of completely depancreatized dogs maintained with insulin has been demonstrated in this laboratory (1). When such animals are fed a diet adequate in respect to calories, proteins, salts, and vitamins, but lacking in raw pancreas, there occurs a fall in all blood lipid constituents (2). The presence of raw pancreas in the diet not only prevents the fall in blood lipids, but also effects a rise to a level far in excess of the normal or preoperative value. Moreover, the addition of the glandular tissue at a time when a lowered level has been established leads to a rapid rise in blood lipids, the values finally attained being again in excess of the normal. Thus, by the removal and addition of raw pancreas in the diet, the blood lipids of completely depancreatized dogs can be made to undergo wide fluctuations.

In the present report a detailed study has been made of the action of pancreatic tissue upon the lipid metabolism of the liver of completely depancreatized dogs. It has previously been established that when the latter are kept alive with insulin and a diet lacking in pancreas, yet containing all constituents in larger amounts than are known to be essential for the normal dog, a marked infiltration of liver lipids occurs soon after pancreatectomy (3).

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EXPERIMENTAL

The care and treatment of completely depancreatized dogs maintained in this laboratory have been previously described (2). In order to promote a better nutritional state, the amount of meat per meal was increased to 280 gm. and the sugar reduced to 50 gm. after September 1, 1935. The animals received their food and were injected with insulin twice daily, at 8.00 a.m. and 4.00 p.m. Blood and liver were taken for analysis between 8.00 and 9.00 a.m.; the dogs had received their last injection of insulin and their last meal at 4.00 p.m. of the previous day. This state of the animal, in which it has been deprived of both food and insulin for 16 hours, is here referred to as the postabsorptive state.

In every case the entire liver was used for lipid analysis. It was removed after the animal had been anesthetized with sodium amytal. The method of sampling the liver and the procedures employed for lipid analysis of liver and blood have been noted elsewhere (2, 3).

A careful search for pancreatic tissue was made in all depancreatized dogs at necropsy. The completeness of pancreatectomy in all animals used in this study was verified.¹

*Relation of Time after Pancreatectomy to Lipid Content of Liver—*Although a lipid infiltration in the liver (3) had already been noted in depancreatized dogs receiving insulin and a diet adequate in all nutritional requirements though lacking pancreatic tissue, two factors remained to be determined: (1) the time of onset of these fatty changes, and (2) the fat content of the liver at various intervals during long periods of survival. An accurate determination of both factors seemed essential in order that the preventive as well as the curative action of various substances might be accurately gaged. Table I records the lipid content of the livers of thirty dogs removed at intervals between 3.5 weeks and 5.5 years² after pancreatectomy. All animals were in good nutritional state at the time they were sacrificed for liver analyses.

¹ We are indebted to Dr. C. L. Connor and Dr. G. R. Biskind of the Division of Pathology for examination of all tissues in this study.

² The recent claim of Dragstedt *et al.* (4, 5) that completely depancreatized dogs ingesting no pancreatic tissue survive for only 2 or 3 months is contrary to previous findings, for it has been shown in this laboratory that,

The results obtained in this study show that, although 32 per cent of fatty acids may appear in the liver as early as 3.5 weeks after pancreatectomy, the rate of lipid infiltration is highly variable. Thus, while Dog D-M showed 32 per cent of lipids in its liver 9 weeks following pancreatectomy, three other dogs maintained under identical conditions for the same period contained between 6.6 and 11 per cent of lipids in their livers. A similar variation was observed at the other time intervals studied. Values as low as 6.6 per cent were observed as late as 14.5 weeks after excision of the pancreas (Dog D-47). Despite these variations, however, it should be noted that all livers contained more than 15 per cent of total lipids when these dogs had been maintained for 19.5 to 36 weeks. The values found at this time ranged from 16.1 to 40 per cent in eight dogs.

The residual fatty acids show that the major part of the increase in lipids is due to deposition of triglycerides. The free cholesterol of the liver is fairly constant, irrespective of the degree of lipid infiltration. Esterified cholesterol, however, remained at normal levels in livers containing up to 10 per cent of fatty acids. In those organs in which the fatty acid content rose above this value, the percentage of cholesterol esters showed a distinct rise. No direct relation was observed between the degree of lipid infiltration and the phospholipid content of the thirty livers.

The observations on Dogs D-A, D-B, and D-C are of prime importance in considering the relation of time to the fat content of the liver, for the survival period of these animals is the longest thus far recorded; namely, 5.5, 4.2, and 5.1 years respectively.

At the time the livers were removed for study, these dogs were in good nutritional state, having maintained a vigorous appetite

when completely depancreatized dogs are fed a diet adequate in calories, proteins, salts, and vitamins, they may survive for as long as 5 years. The completeness of the pancreatectomy in two animals that survived for this length of time was confirmed at necropsy. In passing, it should be noted that the failure of Dragstedt's dogs to survive may have been due to nutritional deficiencies, for the premortal manifestations, namely loss of appetite, loss of weight, apathy, and muscular weakness, are strongly suggestive of vitamin B₁ deficiency (6). Because of faulty absorption resulting from loss of pancreatic juice, it has been the practise in this laboratory to supply large amounts of vitamins of tested potency.

TABLE I

Relation of Time after Pancreatectomy to Lipid Content of Livers of Completely Depancreatized Dogs Maintained with Insulin

The values are expressed on the basis of fresh tissue.

Dog No.	Weight	Period depan- creatized	Liver		Cholesterol				Total lipid	Total fatty acids	Phospholipid	Residual fatty acids*
			Weight	Per cent of body weight	Total	Free	Ester					
							per cent	per cent				
	kg.	wks.	gm.									
D-100	6.6	3.5	315	4.8	0.31					11.0		
D-106	4.0	3.5	225	5.6						31.8		
D-1†	11.5	4.5	480	4.2	0.27	0.10	0.17	63	20.9	18.6		
D-105	9.5	6.5	525	5.5	0.57					7.08		
D-M	9.7	9	700	7.2	0.56	0.17	0.39	70	32.2	29.0	1.07	28.0
D-39	6.0	9	280	4.7	0.25	0.19	0.06	24	8.04	6.93	1.56	5.85
D-40	6.0	9	400	6.7	0.22	0.15	0.07	30	6.57	5.58	1.42	4.58
D-41	6.6	9	355	5.4	0.25	0.19	0.06	24	10.9	10.0	1.67	8.84
D-103	7.2	9.5	327	4.5	0.54					21.6		
D-34	7.4	10	395	5.3	0.30	0.13	0.17	56	30.9	28.7	1.48	27.6
D-N	7.1	10	770	10.8	0.31	0.17	0.14	45	45.7	43.2	1.02	42.4
D-33	8.8	11	485	5.5	0.35	0.18	0.17	49	31.6	28.2	1.74	26.9
D-44	4.6	11	235	5.1	0.32	0.19	0.13	41	15.4	13.6	2.22	12.0
D-35	7.8	11.5	390	5.0	0.31	0.17	0.14	44	15.3	14.0	1.53	12.9
D-38	7.3	12	490	6.7	0.20	0.16	0.04	20	7.96	6.85	1.52	5.80
D-47	9.8	14.5	375	3.8	0.22	0.18	0.04	18	6.55	5.28	1.69	4.12
D-94	4.8	14.5	260	5.4	0.25				12.1	10.6		
D-45	5.5	15	335	6.1	0.22	0.17	0.05	23	9.47	8.35	1.50	7.31
D-58	6.9	15	750	10.9	0.28	0.18	0.10	36	43.5	41.4	1.15	40.6
D-59	8.5	19.5	360	4.2	0.60	0.19	0.41	69	31.0	28.5	1.86	27.0
D-56	7.0	20	385	5.5	0.76	0.17	0.59	78	22.9	21.0	1.40	19.6
D-79	6.0	20	290	4.8	0.38	0.18	0.20	53	27.3	25.2	1.60	24.0
D-84	9.0	20.5	650	7.2	0.38	0.22	0.16	42	31.1	29.2	1.08	28.4
D-52	7.0	21			0.51	0.20	0.31	61	16.1	14.4	1.47	13.2
D-H	9.6	25	690	7.2	0.94	0.11	0.83	88	40.0	36.1	1.08	34.8
D-K	7.0	34	565	8.1	0.40	0.10	0.30	75	29.7	26.3	1.03	25.4
D-85†	9.1	36	540	5.9	0.39				30.2	27.2		
		gra.										
D-B†	7.0	4.2	400	5.7	0.22	0.21	0.01	5	3.52	2.46	1.48	1.46
D-C	10.7	5.1	480	4.5	0.28	0.25	0.03	11	3.93	2.71	2.12	1.27
D-A	7.5	5.5	565	7.5	0.25	0.24	0.01	4	5.60	4.90	1.30	4.02

* Fatty acids other than those in combination with cholesterol and phospholipids (2).

† Male; all other dogs are females.

‡ Received double portions of meat for last 16 weeks.

throughout their stay in the laboratory. The finding of a low percentage of lipids in their livers is indeed striking, the more so since in two of the animals, Dogs D-B and D-C, values well within the normal range were observed. But the total amount of fat contained in the livers of the animals was still in excess of the normal, for in all three the livers remained tremendously enlarged.³ Thus Dogs D-A, D-B, and D-C, weighing 7.5, 7.0, and 10.7 kilos, contained 565, 400, and 480 gm. of hepatic tissue respectively. It was previously shown that the largest liver in a series of normal dogs weighing up to 14 kilos did not exceed 300 gm.

There can be no doubt that the low lipid percentage in the livers of Dogs D-A, D-B, and D-C represents a return to normal in livers that once had been fatty, rather than a failure to have developed at some earlier time a fatty acid concentration greater than that shown at necropsy. This is borne out by the great enlargement of the livers despite their normal or nearly normal lipid percentage; such enlargement undoubtedly occurred as a result of a previous fatty infiltration. 2 years before removal of the liver, Dog D-A showed bile in the urine, the presence of which suggests impaired liver function, a not uncommon accompaniment of fatty infiltration in such dogs. Additional evidence for this view is provided by the finding of extensive fibrotic changes in the livers at the time of removal for lipid analysis. Table I thus brings out what apparently was not recognized previously, that, although fatty livers appear early and remain for a considerable time, spontaneous regression may nevertheless occur in the livers of completely depancreatized dogs when survival is sufficiently prolonged.

The question now arises at what time spontaneous regression of fatty livers occurs. It was noted above that the rate of lipid infiltration that follows pancreatectomy is subject to considerable variation; similar variations undoubtedly occur in the time of initiation of the lipid regression. Small changes in the lipid concentration obviously cannot be detected, particularly in view of the variability of the lipid content of the liver at any given interval after pancreatectomy; hence the exact time when spon-

³ The livers of Dogs D-A, D-B, and D-C showed extensive periportal fibrosis with irregular lobulation indicative of cirrhosis.

taneous cures set in cannot be determined at present. It should be noted, however, that a fatty liver was found as late as 39 weeks after pancreatectomy (Dog D-24, Table II), and in an animal that had received the daily insulin and dietary treatment outlined above, 20 per cent of total lipids was found in the liver

TABLE II

Preventive and Curative Effect of Raw Pancreas upon Fatty Infiltration of Livers of Completely Depancreatized Dogs Maintained with Insulin

The values are expressed on the basis of fresh tissue.

Dog No.	Weight	Period depancrea- tised	Period receiv- ing raw pancreas*	Liver		Cholesterol				Total lipid	Total fatty acids	Phospholipid	Residual fatty acids
				Weight	Per cent of body weight	Total	Free	Ester					
	kg.	wks.	wks.	gm.		per cent	per cent	per cent	per cent of total	per cent	per cent	per cent	per cent
D-11	9.9	11	11	590	6.0	0.21	0.16	0.05	24	3.88	2.64	1.75	1.43
D-5	10.7	10	10	500	4.7	0.22	0.19	0.03	14	5.04	2.66	2.25	1.13
D-16	7.1	10.5	10.5	275	3.9	0.23	0.19	0.04	17	4.06	2.84	2.23	1.31
D-18	9.2	11	11	415	4.5	0.25	0.24	0.01	4	4.79	3.50	2.60	1.75
D-20	8.7	20	Last 4	490	5.6	0.39	0.20	0.19	49	33.5	29.2	2.26	27.5
D-25	10.8	18.5	" 5	850	7.9	0.31	0.18	0.13	42	26.4	23.1	1.81	21.8
D-P	7.4	49	" 5.5†	480	6.5	0.31	0.21	0.10	32	18.4	16.1	2.49	14.4
D-24	9.1	39	" 5.5	800	8.8	0.55	0.21	0.34	62	30.3	26.7	1.52	25.4
D-12	8.5	34.5	" 6	420	4.9	0.27	0.21	0.06	22	13.2	11.3	2.36	9.7
D-61	9.5	32.5	" 15	490	5.2	0.33	0.19	0.14	42	10.4	8.6	1.86	7.2
D-66	7.9	36	" 16	500	6.3	0.28	0.23	0.05	18	12.9	11.5	2.50	9.8

* 125 gm. per meal.

† The animal received no glandular tissue in the diet for the 20 weeks preceding the inclusion of raw pancreas in the diet. Prior to this time the dog received raw pancreas in the diet for the first 10.5 weeks and again from the 20th to the 23rd week after pancreatectomy.

in the postabsorptive state as late as 3 years after pancreatectomy (3).

Preventive and Curative Action of Raw Pancreas—The effect of this glandular tissue on the infiltration of fat in the liver is shown in Table II. In the case of the first four dogs recorded, 250 gm.

of the raw pancreas were fed daily in addition to the usual amounts of meat, sugar, bone ash, and vitamin supplements contained in all mixtures fed after pancreatectomy. In the case of the last seven dogs, the feeding of a similar amount of the untreated glandular tissue was begun at a time when, as shown by Table I, fatty livers had already been established. The livers of the first group were removed from 10 to 11 weeks after pancreatectomy, and in all four animals normal or approximately normal lipid percentages were found, namely 3.9 to 5.0 per cent.

The curative action of the raw glandular tissue on the lipid infiltration is by no means so striking as its preventive action. In Dogs D-20, D-25, D-P, and D-24, all of which had received raw pancreas for the last 4 to 5.5 weeks of their stay in the laboratory (extending from 18.5 to 49 weeks before they were sacrificed), variable degrees of fatty infiltrations were still present. In only three of the dogs recorded in Table II is there an indication that curative action had set in, and in two of them the glandular tissue had been fed for as long as 15 to 16 weeks. In view, however, of the more definite action of autoclaved pancreas (to be shown below), there can be little doubt that the lower lipid values in Dogs D-12, D-61, and D-66 (as compared with Dogs D-20, D-24, D-25, and D-P) represent the partial curative action of the pancreas. Table II thus brings out the fact that, although raw pancreas readily prevents the onset of the fatty infiltration when administered immediately and daily after pancreatectomy, its curative action is a slow process, and a feeding period in excess of 16 weeks is required before complete return to a normal lipid content can be effected.

Preventive and Curative Action of Autoclaved⁴ Pancreas—Dogs D-10 and D-28 (Table III) received 250 gm. of autoclaved pancreas daily in addition to the regular diet during their whole period of maintenance (24.5 and 12.5 weeks respectively) following pancreatectomy. It may be observed that fatty livers failed to appear in both dogs. The total lipid content, as well as the

⁴ Pancreas was cut into small pieces. Portions of 125 gm. were placed in large flasks and autoclaved for 30 minutes at 20 pounds of steam pressure. The entire contents of a single flask were mixed with the other dietary constituents, namely meat, sugar, bone ash, and vitamins, at the time of feeding.

cholesterol, remained within normal limits in their livers. The curative action of the autoclaved tissue was studied in seven dogs, in which the period of life after pancreatectomy varied from 15 to 50 weeks before they were sacrificed. In these dogs the addition of the autoclaved pancreas was begun at a time when,

TABLE III

Preventive and Curative Effect of Autoclaved Pancreas upon Fatty Infiltration of Livers of Completely Depancreatized Dogs Maintained with Insulin*

The values are expressed on the basis of fresh tissue.

Dog No.	Weight	Period depancreatized	Period receiving autoclaved pancreas†	Liver		Cholesterol				Total lipid	Total fatty acids	Phospholipid	Residual fatty acids
				Weight	Per cent of body weight	Total	Free	Ester					
	kg.	wks.	wks.	gm.		per cent	per cent	per cent	per cent of total	per cent	per cent	per cent	per cent
D-10	8.1	24.5	24.5	365	4.5	0.22	0.21	0.01	5	4.20	2.90	1.86	1.64
D-28	7.2	12.5	12.5	320	4.5	0.21	0.20	0.01	5	4.17	2.94	2.50	1.26
D-32	7.8	15	Last 7	500	6.4	0.32	0.17	0.15	47	18.6	17.1	1.99	15.7
D-31†	9.6	14.5	" 9.5	425	4.4	0.44	0.17	0.27	61	28.8	25.7	1.45	24.5
D-O	10.5	50	" 10.5‡	400	3.8	0.23	0.22	0.01	4	4.07	2.65	2.33	1.08
D-60	7.5	35.5	" 15	385	5.1	0.24	0.23	0.01	4	7.95	6.66	2.02	5.30
D-62	5.7	34.5	" 15	240	4.2	0.26	0.24	0.02	8	4.29	3.08	2.26	1.55
D-65†	4.7	35	" 15	270	5.7	0.34	0.24	0.10	29	12.4	11.5	2.12	10.0
D-64	6.2	36	" 16	280	4.5	0.22	0.21	0.01	5	3.71	2.36	2.02	1.00

* Autoclaved for 30 minutes at 20 pounds pressure.

† 125 gm. per meal.

‡ Did not finish meals completely.

§ The animal received no glandular tissue in the diet for the 22.5 weeks preceding the inclusion of autoclaved pancreas in the diet. Prior to this time the dog received raw pancreas in the diet for the first 10.5 weeks and again from the 13th to the 16th week after pancreatectomy.

as judged by Table I, fatty livers had already developed. Although variable results were obtained, there can be no doubt that the autoclaved tissue possessed curative action on the lipid infiltration. Thus in Dog D-O, which received the autoclaved pancreas for the last 10.5 weeks of its 50-week period of maintenance, the lipid content of the liver was 4.1 per cent. More

consistent results were obtained in the group of dogs that were fed autoclaved pancreas for 15 to 16 weeks, *i.e.* for approximately the second half of their period of maintenance after pancreatectomy. In these dogs liver lipids were present to the extent of 8.0, 4.3, 12, and 3.7 per cent.

It is interesting to note that, when the infiltration of fatty acids is prevented by means of raw or autoclaved pancreas, there occurs no rise in the cholesterol ester content of the liver. During

TABLE IV

Effect of Ingestion of Autoclaved Pancreas upon Whole Blood Lipids of Completely Depancreatized Dogs Maintained with Insulin*

Dog No.	Weight	Period depancreatized	Period receiving autoclaved pancreas	Cholesterol				Total fatty acids	Phospholipid	Total lipid	Residual fatty acids
				Total	Free	Ester					
						mg. per 100 cc.	mg. per 100 cc.				
	kg.	wks.	wks.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	per cent of total	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
D-28	7.9	Preoperative		148	111	37	25	340	327	488	94
	7.2	12.5	12.5	143	97	46	32	396	402	539	93
D-10	9.1	Preoperative		162	110	52	32				
	8.1	8	8	141	106	35	25				
	7.7	13.5	13.5	174	129	45	26				
	8.1	24.5	24.5	144	107	37	26	430	349	574	169
D-65		Preoperative		142	93	49	34	350		492	
	4.7	35	Last 15	137	121	16	12	390	344	527	148
D-32	11.5	Preoperative		151	107	44	29	353	308	504	115
	7.6	8		137	105	32	23	332	286	469	117
	7.8	15	Last 7	127	96	31	24	372	357	499	110

* Autoclaved for 30 minutes at 20 pounds pressure.

the regression of fatty livers effected by the untreated and autoclaved tissue, the cholesterol ester content of the liver returns to normal.

Effect of Autoclaved Pancreas on Blood Lipids—The striking effects of raw pancreas upon the level of the blood lipids has already been noted (1). To study the influence of autoclaved pancreas, the feeding of it was begun at three different intervals: (1) immediately after pancreatectomy; (2) from 2 to 5 months after pancreatectomy (here a lowered lipid concentration in the

blood was present at the time when the feeding of the autoclaved tissue was initiated); (3) from 5 weeks to 3 months after pancreatectomy, during which time the animals received raw pancreas (here autoclaved pancreas was substituted for the raw glandular tissue at a time when a lipid level above normal had been established).

In Dogs D-10 and D-28 (Table IV) the feeding of autoclaved pancreas was begun immediately after pancreatectomy and was continued daily for 24.5 and 12.5 weeks, respectively, following the operation. No striking change was observed in any of the lipid constituents. But it should be noted that, although the

TABLE V

Effect of Substitution of Autoclaved for Raw Pancreas upon Whole Blood Lipids of Completely Depancreatized Dog D-70 Maintained with Insulin

Interval since pancreatectomy	Weight	Duration of raw pancreas* in diet	Duration of auto-claved pancreas* in diet	Cholesterol				Total fatty acids	Phos-pho-lipid	Total lipid	Re-sidual fatty acids
				Total	Free	Ester					
days	kg.	days	days	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	per cent of total	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
52	8.6	52		231	137	94	41	522	417	753	173
60	8.7	60		242	130	112	46	503	479	745	100
68	8.7		8	184	117	67	36	403	395	587	89
79	8.5		19	161	117	44	27	518	421	679	204
87	8.4		27	153	126	27	18	542	406	695	250
94	9.0	7		231	141	90	39	529	484	760	139
124	8.8	37		294	145	149	51	505	487	799	69
135	9.0	48		244	140	104	43	555	490	799	151

* 125 gm. per meal.

rise above normal to the extent observed with the raw tissue did not occur, the feeding of the autoclaved pancreas did prevent the cholesterol level from falling appreciably below normal. This is clearly shown in Dog D-10, in which cholesterol ester values between 35 and 45 mg. per cent were found during the 6 months after pancreatectomy, as compared with a preoperative value of 52 mg. While cholesterol esters remained close to the preoperative level, total fatty acids and phospholipids rose to some extent.

Dogs D-65 and D-32 received autoclaved pancreas for the latter half of their periods of maintenance after pancreatectomy, while no glandular tissue was included in the diet fed during the first half. Total and esterified cholesterol did not rise above the pre-operative levels. Total fatty acids and phospholipids showed a slight but definite elevation.

A typical result of the effect of substituting autoclaved pancreas for raw pancreas in three dogs in which a high lipid level in the blood stream had been established by feeding the raw tissue is shown in Table V.⁶ Dog D-70 was fed raw pancreas for the first 60 days after pancreatectomy, and at the end of this time total lipids attained a value of 745 mg. per cent, 242 mg. of which were total cholesterol. For a period of 27 days autoclaved pancreas was then substituted for the raw tissue and the blood lipids examined at three intervals. Despite the ingestion of autoclaved pancreas, a rapid and progressive fall in cholesterol took place. In a period of 8 days esterified cholesterol decreased from 112 to 67 mg. per cent; 19 days later this constituent dropped to 27 mg. per cent. The changes in total fatty acids and phospholipids were not so striking as those in cholesterol. Phospholipids were affected to a small extent, whereas total fatty acids underwent even less change. Raw pancreas was now substituted for the autoclaved tissue, and within 1 week the concentration of total cholesterol increased by 78 mg. per 100 cc. of blood, while the esterified portion rose 200 per cent.

DISCUSSION

The present study reveals two characteristics of the livers of completely depancreatized dogs maintained with insulin and a diet adequate in calories, proteins, salts, and vitamins but containing no raw pancreas: (1) the existence of a time factor in the development of massive lipid deposits, and (2) the variability of this response.

Fatty livers are not always present. In any given dog a certain interval of time after pancreatectomy is necessary to insure the development of an extensive infiltration of fat. Although as much as 32 per cent of fatty acids may appear in the liver as

⁶ A progress report containing other data has appeared (7).

early as 3.5 weeks after excision of the gland, it is nevertheless necessary to recognize that a fatty acid content as low as 5.3 per cent was observed as late as 14.5 weeks after pancreatectomy. Under the conditions of the present investigation, which involved a study of thirty animals, it required approximately 20 weeks for livers to attain a fatty acid content in excess of 14 per cent. Once attained, fatty livers apparently remain for long periods, and in a single dog such a liver was observed as late as 3 years after pancreatectomy (3). If, however, the animals survive long enough, a spontaneous decline in the fat content of the liver may occur. In three dogs that had survived from 4.2 to 5.5 years, approximately normal percentages of fatty acids were found, although the total amount of fatty acids present was still in excess of the normal, owing to the fact that the size of the liver failed to regress as the fat left it.

Pancreas can cure as well as prevent these lipid changes in the liver. A striking feature of its curative effect is its slow action. While the ingestion of 250 gm. of the raw glandular tissue each day following pancreatectomy (*i.e.* from a time when the fat content of the liver was still normal) completely inhibited the deposition of abnormal amounts of fat in the liver, the administration of the same amount of raw pancreas for as long as 16 weeks, beginning at a time when fatty livers had been established, failed to empty the liver completely of its abnormal amounts of fat. A feeding period as short as 4 or 5 weeks apparently had no appreciable effect on fatty livers. Under the conditions of the present study raw pancreas must be fed for a period longer than 16 weeks if the fat content of the liver is to be restored to normal. The active factor (or factors) is heat-stable, for after being autoclaved for 30 minutes at 20 pounds pressure pancreatic tissue still retained its preventive as well as curative action. This observation would seem to rule out an enzyme nature for the liver factor.

On the other hand, the substance in raw pancreas effective in raising the blood cholesterol above normal is heat-labile. This is most clearly shown in the experiments in which autoclaved pancreas was substituted for raw pancreas at a time when the latter had established a high lipid level. The treated tissue failed to sustain the raised cholesterol level, and a rapid drop in

this constituent soon followed the replacement of the raw for the autoclaved tissue. It is interesting to note, however, that when the deposition of abnormal amounts of fat in the liver is inhibited by the ingestion of autoclaved pancreas, there is no drop in the blood lipid level such as is usually observed when none of the glandular tissue is fed.

During the progress of this work, Dragstedt *et al.* reported the isolation of a factor in the pancreas capable of curing fatty livers in depancreatized dogs maintained with insulin (4, 5). Their observations that have not been confirmed will now be considered in the light of the present investigation.

1. Dragstedt *et al.* ((4) p. 122) claim that "the daily administration of 1000 gm. of beef pancreas that had been boiled for 15 minutes did not prevent the development of typical fatty changes in the liver." This is not borne out by the results of the present study. It was found in this laboratory that pancreas subjected to 20 pounds of steam pressure in an autoclave for 30 minutes still retained its curative and preventive action on fatty livers. This is shown in Table III. Thus, 24.5 weeks after pancreatectomy, during which time Dog D-10 had received daily 250 gm. of autoclaved pancreas, the liver contained 2.9 per cent of fatty acids.

2. Dragstedt *et al.* report curative action with extracts corresponding to 100 gm. of the fresh tissue, in as short a time as 3 to 6 weeks. The results obtained in the present study, however, make it clear that the curative action of the raw tissue is a slow and irregular process. In three dogs that had received 250 gm. of the raw glandular tissue for 4 to 5.5 weeks after fatty livers had been established, 29, 27, and 23 per cent of fatty acids were still found in mixed samples of livers (Dogs D-20, D-24, and D-25 in Table II).

The above differences may be ascribed to the method of assay employed by Dragstedt *et al.*, the accuracy of which may be questioned (8). Their conclusions are based on comparisons of the fat content⁶ of small sections of liver removed at biopsy at various intervals related to extract feeding. In our preceding report it was shown that low lipid as well as fatty sections were

⁶ It is important to note that chemical methods were not employed by Dragstedt *et al.* for lipid determinations. Fat estimations were made from the appearance of livers stained with scharlach R.

removable from a single liver. In view of this patchy distribution—the occurrence of which is not predictable in a liver—it is difficult to accept results obtained by a method of assay involving histological examination of small biopsy samples. This is of particular importance in the study of the curative action for short intervals (*e.g.* 3 to 6 weeks) in which but small changes are to be expected. The extreme differences in the lipid content of several sections removed from a single liver might well surpass the absolute percentage decrease shown by analysis of a mixed liver sample.

The striking effect of raw pancreas on the lipid metabolism of the liver of the depancreatized dog has been known for a long time, and the question may be asked, to what factor in pancreas is this action due? Best and his coworkers (9) attribute it to the choline content of pancreas, while Dragstedt *et al.* (5) attribute it to a new hormone present in this tissue. In view of the unsatisfactory method of assay employed by Dragstedt *et al.*, evaluation of their claim for a new hormone must await a more rigid proof. While no attempt is here made to deny the existence of a new hormone, it yet seems necessary to call attention to the spontaneous regressions in fatty livers observed when depancreatized dogs survive long enough. The disappearance of fat in animals that have received none of the glandular tissue for as long as 5.5 years lends doubtful support to the claim for a new hormone whose action is to prevent the deposition of abnormal amounts of fat in the liver. It is also necessary to call attention to the level of the protein intake, since Channon and Wilkinson (10) have shown that a low protein intake is associated with the production of fatty livers in rats. It is well known that digestion and absorption of proteins are interfered with by excision of the pancreas.

SUMMARY

1. The relation of time after pancreatectomy to the development of fatty livers was studied in thirty completely depancreatized dogs maintained with insulin and a diet adequate in calories, proteins, salts, and vitamins. Although fatty livers may appear as early as 3.5 weeks after excision of the gland, the occurrence of fatty livers is not a constant finding at this time interval.

Livers containing as little as 6.6 per cent fat were observed as late as 14.5 weeks after pancreatectomy. It required a period of at least 16 weeks to insure a consistent finding of fatty acids in excess of 14 per cent in the livers of completely depancreatized dogs.

2. If completely depancreatized dogs survive long enough, spontaneous regressions of the fatty livers may occur, and this despite the absence of raw pancreas in all diets fed during the entire period of survival.

3. The ingestion of raw pancreas prevents the infiltration of fat in the livers of depancreatized dogs. This glandular tissue also possesses curative action on livers in which large amounts of fat have been deposited.

4. The factor in raw pancreas active in preventing and curing fatty livers is heat-stable, for it is shown that, after being autoclaved at 20 pounds of steam pressure for 30 minutes, pancreas still retains its curative and preventive action.

5. The factor in pancreas which, when it is ingested by completely depancreatized dogs being maintained with insulin, produces an elevation in blood lipids above the normal is heat-labile. This is destroyed when subjected to 20 pounds of steam pressure for 30 minutes.

6. The nature of the active factors in pancreas is discussed.

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EFFECT OF ACTIVITY ON THE PHOSPHOLIPID AND CHOLESTEROL CONTENT OF MUSCLE

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In earlier papers evidence was presented of a relationship between the specific activity of a tissue and its content of phospholipid. For example, in organs with cyclic activity such as the corpus luteum (1) and mammary gland (2), it was found that the phospholipid content increased greatly with activity of the gland, remained high during its activity, and diminished as the activity fell off. During regression, in the case of the corpus luteum, the phospholipid was apparently replaced by fat and cholesterol esters. Boyd and Elden (3) have shown that estrin production in the corpus luteum is proportional to the phospholipid content. Yasuda and Bloor (4) found that the phospholipid percentage of malignant, fast growing tumor tissue was 2 to 3 times that of benign tumors. The cholesterol content changed similarly to the phospholipid content, but the increase was not as great. In work on tumors in this laboratory, Haven (5) found a much higher phospholipid percentage in the outside, actively growing portion of malignant rat tumors than in the inside, slow growing or recessive and sometimes necrotic portion. The interior of the tumor also contained much more cholesterol ester than the outside part. Boyd (6) found differences in the phospholipid content of white blood cells, which he was able to correlate with differences in their activity as phagocytes. The chances of recovery of human beings in cases of severe infections varied with the phospholipid content of the white blood cells. The higher their phospholipid content, the better the chances of recovery. In beef muscle, Bloor and Snider (7) found a correlation between the activity of the muscle and its phospholipid con-

tent. The more active the muscle, the higher its phospholipid content.

In the belief that muscle, because of its single type of activity, offered a particularly good experimental tissue for testing the hypothesis of a positive relation between activity and phospholipid content, these authors continued the investigation on a variety of muscles from many animals, some wild and capable of great activity, some domesticated and sedentary (2). It was found without exception that the more used muscles had a higher phospholipid content than the less used ones. The differences in cholesterol content were in the same direction but not invariable and not as great, so that the phospholipid to cholesterol ratio was generally higher in the more used muscles.

However, Ludewig and Chanutin (8) have recently pointed out that this evidence was indirect; *i.e.*, the differences found were those which occurred naturally and not those which were experimentally produced. These workers experimentally increased the activity of the kidney of rats by removal of a large part of the kidneys and indirectly the activity of the heart by the resultant hypertension. Although considerable hypertrophy of both kidney and heart was produced by the extra load, the phospholipid and cholesterol percentage of the hypertrophied tissue was the same as that of the controls. These results led them to doubt the hypothesis of the relation of activity to phospholipid content.

Without questioning their findings, part of which have been reproduced in this laboratory (see below), it may be noted that both heart and kidney *hypertrophied* in response to the extra work. It might, therefore, be argued that the increased amount of tissue had taken up the extra load, each unit weight of tissue doing the same amount of work as before, other adaptive change being therefore unnecessary. There is also the possibility that the same substances are not under consideration. Ludewig and Chanutin determined their phospholipid by calculation from ether-soluble phosphorus, while in our work phospholipid was determined directly by combustion of the acetone-insoluble lipid. It has been shown that calculations of phospholipid from lipid phosphorus always give high results (9, 10), owing to the inclusion of phosphorus-containing compounds other than phospholipid. Calculated to phospholipid (lipoid P \times 25), the values obtained

by Ludewig and Chanutin are high. Thus their values for heart phospholipid are 2.8 to 3.1 per cent of moist weight in both control and experimental animals and for kidney 3.3 to 3.7 per cent in both. Our values on moist weight of rat heart (see Table III) average 1.7 for the resting and 1.94 for the exercised rats. Only one animal in our series gave a value as high as the lowest of their values. For rat kidney, unpublished results from our laboratory give average values of 2 per cent. If the phospholipid values are truly represented by the percentages given by Ludewig and Chanutin, the content is already at a high point for that tissue and would not readily increase.

In the case of hypertrophy of the kidney, we can confirm the results of Ludewig and Chanutin by unpublished work done on kidneys made hypertrophic (about 11 per cent) by the high protein diet of MacKay *et al.* (11), in which case it was found that the phospholipid percentage of the hypertrophic kidneys was essentially the same as in that of the controls. As regards muscle, some examples of work hypertrophy without increase of phospholipid are given below.

EXPERIMENTAL

In the present paper is contained additional evidence bearing on the effect of activity on muscle phospholipids. This evidence is of two kinds: (a) the result of "natural" differences in extent of activity of the same muscles in different animals under different natural conditions, and (b) the result of experimentally produced differences in activity in heart and other muscles of controlled animals.

Natural Differences in Muscle Activity

Atrophic or Dystrophic Muscles from Various Sources—Eight muscles of chickens dead of "range paralysis," an epidemic disease characterized by progressive paralysis followed by death from inanition, were examined by methods already described (12),¹

¹ A modification of the procedure which has been in use in this laboratory for some years but which apparently has never appeared in print, and which assumes importance in view of the comment of Man (13) regarding oxidation of the phospholipid during evaporation of the lipid solvent, is as follows: An aliquot portion of the alcohol solution of the muscle lipids is measured

typical results of which are shown in Table I. The average normal values for these muscles in chickens are given in parentheses in Table I.

The lecithin to cephalin ratios in the skeletal muscles of these animals, as determined by Dr. P. L. MacLachlan of this department by the method of Lintzel and Monasterio (14), were found to be reversed; *i.e.*, 46:54 found, 54:46 normal.

The values given in Table II were obtained from atrophic or dystrophic human muscle.

The normal lipid content of human skeletal muscle averages for phospholipid 3 per cent and for cholesterol 0.24 per cent of the dry weight, and the phospholipid to cholesterol ratio is 14.

Muscles Which in a State of Nature Are Very Active—The pectoral muscles of a bat (weight 15 gm., wing area 20 sq. inches) gave values in terms of dry weight as follows: phospholipid 8.8 per cent, cholesterol 0.6 per cent, and a phospholipid to cholesterol ratio of 15. The pectoral muscles were 6.4 per cent of the body weight as compared with 20 per cent in the pigeon and 1 per cent in the rat. The average values of the pectoral muscle of flying pigeons (see Table IV) were 4.56 for phospholipid and 0.24 for cholesterol. The bat pectorals were thus much larger than those of the rat but about one-third of the relative weight of those of the pigeon. Their content of vital lipids (phospholipid and cholesterol) was about twice that of the pigeon pectorals. Hypertrophy in the bat is limited probably because of the small area

into a 100 to 125 cc. Erlenmeyer flask fitted with a light watch-glass which fits into the slightly flared top of the flask and during the evaporation acts as a flutter valve, allowing the escape of the alcohol vapor but preventing the entrance of air. The valve effect is heightened by the film of condensed solvent which forms around the edges of the glass. Evaporation is not carried to dryness but only to absence of alcohol, and there are always 2 or 3 drops of water left in the flask. The petroleum ether is added at once, without cooling the flask, and as a result solution of the lipids takes place quickly. A second treatment with hot solvent leaves only slightly turbid water behind; a third treatment with solvent is made for certainty. The amounts of petroleum ether used are 15, 10, and 10 cc. Under these circumstances the flasks are at all times full of the solvent vapor and no evidence of oxidation has been observed. If the evaporation of the first solvent is allowed to reach dryness, changes take place which prevent complete extraction by the petroleum ether.

of attachment available and also because added weight would be undesirable. Under these conditions, the extra load is compensated for by a very high phospholipid and cholesterol content.

Also, there were examined the thigh muscles of four very active wild mice of 12 to 15 gm. in weight. These gave an average value for phospholipid of 7.7 per cent of the dry weight and for cholesterol of 0.42 with a phospholipid to cholesterol ratio of 19. The

TABLE I
Changes in Chicken Muscle in Paralysis

The average normal values for these muscles are given in parentheses.

Muscle	Phospholipid	Cholesterol	Phospholipid to cholesterol ratio
	<i>per cent moist weight</i>	<i>per cent moist weight</i>	
Ventricles.....	1.2 (1.5)	0.35 (0.12)	5 (14)
Thigh.....	0.75 (0.90)	0.123 (0.07)	6 (16)
Pectoralis major.....	0.33 (0.34)	0.062 (0.04)	5 (8)
Gizzard.....	0.25 (0.60)	0.23 (0.16)	1.1 (4)

TABLE II
Atrophic Changes in Human Muscle

Disease	Muscle	Phospholipid	Cholesterol	Phospholipid to cholesterol ratio
		<i>per cent dry weight</i>	<i>per cent dry weight</i>	
Muscular dystrophy.....	Psoas	0.40	0.40	1
“ “.....	Diaphragm	2.4	0.30	8
“ “.....	Pectoral	2.4	0.58	3
Emaciation (carcinoma).....	Thigh	2.5	1.0	2.5

thigh muscles of the exercised rat (see Table III) had an average phospholipid value of 3.4 per cent, cholesterol 0.24 per cent, and a phospholipid to cholesterol ratio of 14. The adaptations to activity in the mice were the high phospholipid, cholesterol, and phospholipid to cholesterol ratio.

From these results it may be seen that, compared with normal muscles, the atrophic and dystrophic muscles had low phospholipid, high cholesterol, and a low phospholipid to cholesterol

ratio. On the other hand, in agreement with earlier work, very active muscles such as those of the bat and wild mice had very high phospholipid, high cholesterol, and high phospholipid to cholesterol ratios. Of the two lipids, the cholesterol tends to be the more constant, while the phospholipid varies widely with the use of the muscle, so that high phospholipid and a high phospholipid to cholesterol ratio are especially characteristic of active, well nourished muscles, while low phospholipid, high cholesterol, and a low phospholipid to cholesterol ratio characterize little used or atrophic muscles.²

Experimental Differences in Muscle Activity

Exercise and Idleness in Rats—Matched pairs of animals of about 150 gm. in weight were selected and from each pair one was confined in a small cage; the other was put in a similar cage inside an 18 inch exercising wheel made according to Slonaker (15). The wheel was equipped with a counter which would register regardless of whether the wheel was turned forward or backward. After a preliminary learning period most of the rats ran freely, going distances averaging about 5 miles per day. Those rats which did not run freely were exchanged for ones which did, the lazy ones being used to make up part of the controls. After a period of 1 or 2 months running, the animals with their controls were killed, weighed, a series of ten or twelve muscles or muscle groups from each animal was completely removed, weighed, cut up fine with scissors, and samples taken for analysis and the determination of dry weight. The samples for analysis were ground with sand and extracted with hot alcohol; the extracts were made up to volume and analyzed as described in earlier work (12). Ordinarily ten muscles or groups of muscles were analyzed. These were heart (ventricle), neck (vertebral), diaphragm, upper arm, forearm, pectoralis, loin and back, belly

² The accumulation of cholesterol and especially of cholesterol esters together with fat which is found in the atrophic and dystrophic muscles seems to be characteristic of tissues which are going backward metabolically. It is found in the interior of tumors and in the regressive corpus luteum, as noted, and in atheromatous arteries. The accumulation of these substances in liver under abnormal conditions may be part of the same phenomenon. The low phospholipid to cholesterol ratio in inactive muscles probably indicates the beginning of the same process.

TABLE III
Effect of Exercise on Lipids of Rat Muscle

Kind	Condition	Weight			Phospholipid			Cholesterol		
		Average	Range	Stand- ard devia- tion	Average	Range	Stand- ard devia- tion	Average	Range	Stand- ard devia- tion
Heart	Resting	0.302	0.265-0.346	7.3	per cent dry weight			per cent dry weight		
	Exercised % change	0.352 +16.5	0.290-0.418	8.8	6.99 8.08 +15	4.83-8.04 5.25-12.9	10.7 25.3	0.496 0.562 +13.4	0.354-0.562 0.450-1.04	11.8 27.4
Neck	Resting	0.85	0.55-1.52	28.2	3.81	2.73-4.62	13.1	2.96	0.234-0.373	16.9
	Exercised % change	1.03 +21	0.55-1.65	25.2	3.88 +2	2.85-4.65	11.9	2.85 -4	0.250-0.328	8.6
Diaphragm	Resting	0.280	0.211-0.350	16.2	4.04	3.23-4.65	9.5	0.350	0.250-0.498	16.5
	Exercised % change	0.361 +39	0.225-0.967	52.6	3.54 -14	2.31-4.42	18.5	0.296 -18.2	0.181-0.385	18.5
Thigh	Resting	6.41	5.03-9.16	17.6	3.23	3.76-2.69	10.7	0.246	0.192-0.304	15.5
	Exercised % change	7.24 +12.9	5.12-10.6	14.2	3.34 +3.6	2.38-3.85	12.6	0.231 -6.6	0.165-0.292	16.7
Front leg	Resting	2.18	1.49-3.79	31.6	3.42	2.77-3.88	11.0	0.277	0.211-0.338	16.7
	Exercised % change	2.53 +16	1.0-5.17	39.1	3.96 +15.7	3.57-4.38	8.0	0.296 +6.9	0.231-0.365	16.9
Abdominal wall	Resting	3.39	3.08-3.91	7.9	2.81	2.27-3.31	13.1	0.254	0.173-0.331	18.2
	Exercised % change	3.66 +7.9	2.86-5.52	18.0	2.96 +5.5	2.00-3.96	16.6	0.273 +7.6	0.208-0.365	19.7
Gastroc- nemius	Resting	1.09	0.78-1.34	13.8	3.26	2.57-3.85	11.3	0.261	0.211-0.319	12.9
	Exercised % change	1.32 +21.1	0.97-2.12	21.2	3.73 +14.3	3.50-4.08	4.5	0.292 +11.8	0.242-0.418	14.5
Back	Resting	2.51	1.76-3.91	30.3	2.81	2.19-3.38	13.4	0.227	0.146-0.298	18.6
	Exercised % change	2.96 +17.9	2.00-4.47	24.3	3.15 +12.3	2.34-3.61	10.6	0.238 +5.1	0.169-0.323	17.7
Pectoralis	Resting	0.918	0.68-1.19	15.2	3.42	2.61-4.08	13.5	0.285	0.192-0.346	16.2
	Exercised % change	1.0 +8.9	0.64-1.55	24.0	3.73 +9	2.96-4.54	11.3	0.285	0.173-0.346	13.0

wall, thigh, and gastrocnemius. Phospholipid, cholesterol (total), fat, and dry weight were determined. The weight of the muscle in per cent of body weight was calculated in the two groups to reveal hypertrophy resulting from exercise. Fifteen animals in each group were examined and, from the mass of data, the averages, range of values, and standard deviation from the mean were selected and given in Table III.

Flying and Non-Flying Pigeons—Pigeons from hatched pairs were taken at the time when they were beginning to fly. One from each pair was confined in a small cage, the other allowed to fly freely in a large barn. At periods varying from 2 months to a year, pairs were killed and the muscles analyzed. The muscles used were the heart (ventricle), neck (vertebral), pectoralis major, pectoralis minor, upper arm, forearm, thigh, gastrocnemius, abdominal wall, and gizzard. Determinations were made as in the rats, and the values from fifteen flying and thirteen non-flying birds are collected in Table IV.

Results

Rats

Phospholipid—In the exercised animals, phospholipid averaged higher in all the muscles except the diaphragm and notably higher in the heart, front leg, gastrocnemius, and back, all of which are directly concerned with running. Of those muscles in which the increase in phospholipid was small, the thigh, belly wall, pectoralis, and neck, only the thigh muscles would be concerned with running. The only muscle in which the phospholipid averaged lower in the exercised animals was the diaphragm, and in this muscle there was marked (39 per cent) hypertrophy. The spread of phospholipid values in all muscles except the heart and diaphragm was about 10 per cent above or below the average and was the same in both exercised and resting muscles. In the heart, the variation from the average was much greater in the exercised animals (about 3 times the variation in the resting). In both heart and diaphragm in the resting animals, the variation was relatively narrow.

Cholesterol—As has been usually found in muscle, the changes in cholesterol content follow the changes in phospholipid but are less extensive. The changes are positive and of about the same

extent in heart, belly wall, and gastrocnemius; positive but less in the front leg and back; and negative or unchanged in the neck, diaphragm, thigh, and pectoralis. The phospholipid to cholesterol ratio was about 16 per cent higher as the result of exercise in the thigh, front leg, and back and about the same in the other muscles.

Hypertrophy—As shown by the change of muscle weight in per cent of body weight, exercise resulted in a hypertrophy in all muscles. The hypertrophy was greatest in the diaphragm which, as noted, showed a diminished phospholipid and cholesterol content; next in the neck, gastrocnemius, heart, thigh, back, and front leg; and lowest in the belly wall and pectoral muscles, which presumably had less to do with running.

General—The deviations from the average for the phospholipid and cholesterol are about what would be expected of "biological" variation. There are however some exceptional instances which call for comment.

Rat 25 averaged 5.5 miles per day for 59 days. Its heart was 50 per cent over the average heart weight for the animal's size, with a phospholipid content 40 per cent below the average and cholesterol 11 per cent below. The skeletal muscles showed the normal hypertrophy for exercised animals of 10 to 30 per cent and had phospholipid and cholesterol values which were average for the exercised group. The most notable adaptation to the extra work in this animal was the hypertrophied heart.

Rat 27 averaged 5.9 miles per day for 68 days and showed about average exercise values for lipids throughout its muscles. The heart was a little larger than the average and its phospholipid content a little lower. It had the best running record of the lot. The compensations in this animal were evenly distributed throughout the muscles.

Rat 11, with a diaphragm nearly 3 times the average weight, had a heart of average weight containing high phospholipid (+25 per cent) and cholesterol (+10 per cent). The muscles of the front leg and the gastrocnemius were considerably below the average in weight (front leg, 60 per cent; gastrocnemius, 25 per cent) with phospholipid and cholesterol values slightly above the average. The notable compensation in this animal was in the circulo-respiratory system—hypertrophied diaphragm, high heart phospholipid.

TABLE IV
Effect of Exercise on Lipids of Pigeon Muscle

Kind	Condition	Weight			Phospholipid			Cholesterol		
		Average	Range	Stand- ard deviation	Average	Range	Stand- ard deviation	Average	Range	Stand- ard deviation
Heart	Resting	1.08	0.80-1.26	14.8	6.68	5.04-7.64	12.6	0.568	0.400-0.840	16.1
	Exercised	1.01	0.71-1.24	15.8	7.88	6.52-8.72	7.1	0.596	0.420-0.780	15.4
	% change	-6.9			+18			+4.9		
Neck	Resting	0.74	0.52-1.40	33.8	3.45	2.64-4.28	16.0	0.362	2.68-4.46	15.4
	Exercised	0.87	0.63-1.02	14.9	4.32	3.31-7.56	9.7	0.404	3.13-6.96	26.7
	% change	+17.6			+25.2			+11.5		
Pectoralis major	Resting	15.8	12.1-21.2	21.0	3.97	3.13-5.29	17.5	0.233	1.64-3.27	25.4
	Exercised	17.3	11-19.8	11.7	4.56	3.48-5.67	14.5	0.237	1.88-3.48	20.6
	% change	+9.5			+14.9			+1.5		
Pectoralis minor	Resting	3.19	2.0-4.6	23.2	2.89	1.84-3.97	20.5	0.209	0.160-0.275	15.5
	Exercised	2.77	1.6-3.6	18.1	3.41	2.65-3.97	15.7	0.198	0.132-0.247	13.9
	% change	-15.1			+18.1			-5.3		
Upper arm	Resting	2.02	1.3-2.57	22.3	3.31	2.05-4.28	15.8	0.317	0.237-0.505	19.8
	Exercised	1.71	1.17-2.16	15.3	3.44	2.68-4.35	13.7	0.272	0.188-0.334	16.7
	% change	-18.1			+4.2			-16.6		
Forearm	Resting	1.76	1.00-2.57	21.0	3.76	2.86-4.46	11.1	0.390	0.278-0.696	20.2
	Exercised	2.02	1.42-2.24	21.8	4.25	3.55-5.23	12.0	0.313	0.230-0.383	18.9
	% change	+6.04			+12.9			-24.4		
Thigh	Resting	8.15	2.0-2.9	16.7	3.10	1.88-3.76	18.0	0.345	0.212-0.540	12.8
	Exercised	10.23	2.08-4.1	19.7	3.24	2.78-4.38	16.9	0.275	0.174-0.387	22.8
	% change	+25.6			+4.5			-25.3		
Gizzard	Resting	4.49	0.87-1.69	19.7	2.47	1.77-3.27	21.3	0.571	0.313-0.825	26.6
	Exercised	4.42	0.73-1.74	23.6	2.61	1.84-3.31	18.3	0.498	0.278-0.644	21.7
	% change	-1.6			+5.6			-14.7		
Abdominal wall	Resting	2.06	0.36-1.30	22.0	3.55	2.47-0.06	25.5	0.435	0.244-1.22	21.1
	Exercised	1.88	0.40-0.95	24.1	3.69	3.34-4.53	9.8	0.327	0.226-0.408	14.9
	% change	-9.3			+3.9			-32.9		
Gastrocnemius	Resting	4.35	0.91-1.62	18.0	2.79	2.51-3.76	17.0	0.338	0.264-0.376	11.8
	Exercised	4.46	0.81-2.00	29.7	3.48	2.37-4.66	16.0	0.324	0.198-0.435	22.6

Rat 3 with the highest heart phospholipid of all the rats (3.1 per cent, which is 60 per cent above the average), had a heart of average weight for the group and front leg muscles 36 per cent above average in weight with average lipid content. Other muscles were average in weight and composition. It had run 56 days at an average of 5 miles per day. The compensatory changes in this animal were the increased heart phospholipid and cholesterol and hypertrophy of the front leg muscles.

Resting Controls—The highest heart phospholipid value in this group was just under the average for the hearts of the exercised animals, and the lowest was about 15 per cent below that of the lowest exercised heart. In the other muscles there was overlapping of values both for phospholipid and cholesterol. The average variation in body weight was the same in both exercised and resting animals.

To sum up, in rats the effect of exercise showed itself in (a) increased average phospholipid in all the muscles except the diaphragm (which was greatly hypertrophied) and notably in the heart, front leg, gastrocnemius, and back, muscles directly concerned in running. Changes in cholesterol in general followed the phospholipid, but to a less extent, so that the phospholipid to cholesterol ratio was notably higher in the thigh, front leg, and back; (b) hypertrophy of all the muscles examined, most marked in those muscles directly concerned with the exercise—diaphragm, heart, gastrocnemius, thigh, back, and front leg. Of the other muscles, those of the neck were notably hypertrophied, while the belly wall and pectoral muscles were little hypertrophied.

Pigeons

Phospholipid—Increases in phospholipid content over the resting averages were found in all the muscles but were notably great in the heart (18 per cent), neck (25 per cent), pectoralis major (15 per cent), pectoralis minor (18 per cent), and forearm (13 per cent). Except possibly those of the neck, of which the function and behavior in flying are unknown, all these muscles are concerned with flying.

Cholesterol and Phospholipid to Cholesterol Ratio—Notable increases in cholesterol in exercised animals were found only in the belly wall and neck. In the other muscles the changes were

small or negative. Large decreases were noted in the forearm, upper arm, thigh, and belly wall. In all of the muscles, notable increases in the phospholipid to cholesterol ratio were found and these increases were over 20 per cent in the pectoralis minor, forearm, belly wall, and gizzard.

Hypertrophy—The weight of the muscles in per cent of body weight is notably higher (over +10 per cent) in the exercised pigeons only in the neck and thigh. It is notably lower (over -10 per cent) in the muscles of the upper arm (in which the phospholipid to cholesterol ratio is higher owing to lower cholesterol) and pectoralis minor (in which the phospholipid and phospholipid to cholesterol ratio are notably increased). In the pigeons hypertrophy as the result of the flying may, therefore, probably be ruled out, since the only muscles notably above the controls in per cent of body weight were the thigh and neck, and neither of these can with certainty be connected with flight.

The neck muscle is an example of both hypertrophy and increased phospholipid content in the exercised animals; the heart, an example of increased phospholipid content without hypertrophy. The pectoralis major, which supplies most of the energy for flight, showed little hypertrophy, no change in cholesterol, but a considerable gain in phospholipid and, as a result, a notable increase in the phospholipid to cholesterol ratio.

In the pigeons, then, the effects of exercise were (a) increases of phospholipid of 18 to 25 per cent in the muscles concerned with flight, (b) decreases of cholesterol, and (c) increases of phospholipid to cholesterol ratio. Hypertrophy was essentially absent.

DISCUSSION

It should first be noted that according to the usual rules of statistical treatment the results, considered by themselves, are not significant; *i.e.*, the standard deviation from the mean is about equal to the sum of the differences observed. This means that, because of the large natural variations in lipid content of muscle, it is not possible, by this method of interpretation, to distinguish between the effects of exercise and the results of biological variation. Nevertheless, since all but one of the changes in phospholipid are positive and hence in the same direction as

the natural differences found (*e.g.* in earlier work on muscle and in the bat and wild mouse above) and moreover, since, as discussed below, the muscle has other ways of compensating for increased work (hypertrophy, better "servicing" by the circulation, etc.), it is believed that the differences noted are significant and represent another type of adaptation to increased activity. Having a bearing also is the fact that the exercise in both groups of animals was voluntary, was short in duration, and, in the pigeons, was definitely moderate in amount.

A comparison of the muscles in the two groups of exercised animals shows that in both notably higher phospholipid is found in the exercised muscles including the heart. In the rats, there was in addition a hypertrophy of these muscles. In the pigeon, the heart was found to be about 3 times as large in terms of body weight as in the rat, while heart phospholipid average percentage was the same for the corresponding groups of both rats and pigeons with the percentage 15 to 18 per cent higher in the exercised animals. Since the heart in the exercised pigeons did not hypertrophy as the result of the exercise, while in the rats it did, it may be assumed that the large pigeon heart was able to take up the extra load by other adaptations. Both hearts, however, showed increases in phospholipid. In the rat heart there was no relative decrease in cholesterol, while in the pigeon there was. It is reasonable to assume that the change in the lipids in the pigeon heart was an adaptation to the increased activity.

Of the respiratory muscles, the only change in the pigeon (belly wall) was a large decrease of cholesterol with a corresponding increase of the phospholipid to cholesterol ratio. In the rat, the diaphragm was greatly hypertrophied with decreases in both phospholipid and cholesterol.

The significant facts which appear as the result of this study are that the rats responded to exercise by hypertrophy of practically all muscles and by increased phospholipid and phospholipid to cholesterol ratios especially notable in those muscles concerned with exercise, while in the pigeons the response was by increased phospholipid and phospholipid to cholesterol ratios but without notable hypertrophy. The lack of hypertrophy in the pigeons may be due to the fact that in the exercised group the inclination

to fly in the confined space was not great and the amount of exercise was consequently relatively less and also less violent than in the case of the rats.

From these experiments it appears that the effect of exercise on muscle may be either (a) increased phospholipid and cholesterol, (b) decreased cholesterol with or without changes in phospholipid, (c) increased phospholipid to cholesterol ratio, or (d) hypertrophy with or without changes in the lipids.

Dystrophy or atrophy brings about the reverse of these changes; *i.e.*, lowered phospholipid and increased cholesterol percentage, increased phospholipid to cholesterol ratio, and decrease of muscle mass. These changes may also be independent or combined.

As has been discussed in a recent review (16), animals may adapt themselves to increased muscular effort in different ways. If the amount of work performed in a unit of time is high, the used muscles hypertrophy. If, on the other hand, the same increase in work is spread over a longer period, there may be no hypertrophy. In both cases there is an improvement in the circulo-respiratory system consisting largely of a better response by the muscles of the heart and respiration. Changes in the chemical composition of muscle as the result of work are not well established. Increases of the glycogen or of the phosphocreatine or potassium occur only in the earlier period of training and disappear later. Increased buffering capacity and increased muscle hemoglobin are apparently permanent improvements as is also the increased ability to synthesize hexosephosphate.

SUMMARY

In agreement with earlier work, it was found that experimentally increased activity of muscle resulted in increased phospholipid content with less marked changes in cholesterol and a resulting higher phospholipid to cholesterol ratio. These changes while characteristic were not invariable. Other adaptive changes such as hypertrophy sometimes took their place and often accompanied the lipid changes.

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FACTORS INFLUENCING THE DESTRUCTION OF GLUCOSE AND FRUCTOSE BY OXYGEN

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It was noted in a previous paper (1) upon the interconversion of dilute buffered carbohydrate solutions that phosphate apparently influenced the destruction of certain sugars by oxygen. As the oxidation of the sugars was obscured by the conversion of one sugar into another and by the formation of caramel, it seemed desirable to extend the investigation to solutions held at temperatures below their boiling points to decrease these factors. It was found that certain temperatures could be chosen that allowed a considerable destruction of fructose to take place in an atmosphere of oxygen gas, but that reduced the interconversion (Lobry de Bruyn phenomenon (2)) to such a low level that the other reactions taking place were not obscured. This made it possible to study quantitatively the destruction of fructose by oxygen in neutral (buffered) solutions.

The procedure was as follows: Pure sugars from the United States Bureau of Standards were dried over calcium chloride, carefully weighed out, and dissolved in distilled water. These solutions were added to buffer mixtures of known strength and made up to a volume of 25 cc. This mixture was placed in a tube fitted with a condenser and a capillary tube. This entire apparatus was in turn suspended in a large Florence flask also fitted with a condenser. Oxygen or nitrogen gas, first bubbled through distilled water to wash it and to saturate it with water vapor, was introduced into the tube containing the sugar solution through the capillary at a moderate rate. The gas escaped through the condenser on the sugar tube. These precautions reduced to a minimum the loss of water from the solution being studied. A

liquid (or mixture of liquids) whose boiling point gave the desired temperature within the sugar tube (recorded by a small thermometer inside the tube) was placed in the Florence (outer) flask, and was boiled on an electric hot-plate. Samples for analysis were removed from the sugar tube at 3 hour intervals by means of a pipette. The changes in concentration of total reducing substances were determined by the colorimetric copper reduction method of Benedict (3) and variations in the amount of ketose (fructose) present by the resorcinol technique of Roe (4). The

TABLE I

Destruction of Fructose and Glucose in Presence of Oxygen and Nitrogen

In these experiments the temperature was 77.5°; pH 7.0. In each experiment M/15 phosphate buffer was used. "Glucose" figures represent total reducing power determined by Benedict's (3) copper reduction method; "fructose," total ketose present determined by Roe's (4) resorcinol method. The values are expressed in mg. of sugar per 100 cc.

Sugar used.....	40 mg. per cent fructose		40 mg. per cent glucose	
Boiling time	"Glucose"	"Fructose"	"Glucose"	"Fructose"
Experiments with oxygen				
<i>hrs.</i>				
0	38.4	41.0	38.3	0.3
3	30.6	31.5	37.8	0.5
6	24.5	23.4	36.0	0.4
Experiments with nitrogen				
0	40.4	40.6	40.6	0.3
3	38.5	38.3	40.6	0.8
6	39.8	37.2	40.8	1.7

purest glucose and fructose obtainable were used as standards. The pH was determined at room temperature by colorimetric methods.

In order to measure the destruction of fructose by oxygen in the presence of phosphates conditions were chosen under which only slight conversion to glucose took place. Table I shows the results of four experiments in which glucose and fructose solutions of the same initial strength buffered to a pH of 7.0 with phosphate were heated at the same temperature (77.5°) for the same length of

time in the presence of nitrogen and oxygen gases. In the experiments with nitrogen, no destruction took place in either case. Minimal conversion occurred in all four experiments. In the presence of oxygen, there was a marked destruction (39.5 per cent) of fructose. Glucose, similarly treated, showed only a 5.7 per cent destruction. This small amount of destruction was probably due to the slight conversion that occurred and the subsequent destruction of the fructose produced, as is shown by comparison with the experiments with nitrogen gas. The pH in all this group remained at 7.0.

It appeared from previous experiments (1) that the presence of phosphates had a direct influence upon the destruction of fructose by oxygen, apart from its effect on the reaction of the solution. Therefore a series of experiments was carried out in which equivalent fructose solutions buffered to comparable pH values were treated with acetate, borate, carbonate, and arsenate buffers. To compare these different buffer systems to the best advantage various levels of pH were chosen at which the phosphate, as well as the other buffer systems, was effective. This made it possible to use each buffer system at a pH well within its range. As the reaction was made more alkaline, the interconversion and the destruction of fructose both increased. Lowering the temperature, however, decreased the rate of these reactions. Therefore, for each pH level studied the temperature was maintained at a level that gave minimal conversion yet appreciable destruction of fructose in the presence of phosphate.

Table II shows that only in the case of experiments with phosphate and arsenate buffers was there a significant destruction of fructose, although in the others the strength of the buffer solutions, the pH, and the temperature were the same. In all cases the conversion was reduced to a negligible amount, for the values found for fructose by the Benedict and Roe methods were identical within the limits of experimental errors. The buffer solutions were all standard buffers with the exception of the carbonate and the carbonate-phosphate mixtures. These were made by passing a mixture of 70 per cent oxygen and 30 per cent carbon dioxide into a solution containing sodium bicarbonate. The pH was determined in these experiments in the hot solution, colorimetrically, and appeared reasonably constant. This apparent specific effect of

TABLE II
Destruction of Fructose in Presence of Various Buffers

The initial fructose concentration in each experiment was 40 mg. per 100 cc. No conversion took place in the experiments shown here. The values for initial (0 hour) and final (6 hours) concentration of fructose are shown. The figures express mg. of fructose per 100 cc. The per cent destruction is shown in each case.

pH..... Temperature, °C.....	6.0 77.4			7.0 77.4			7.5 84.0			8.0 84.0		
	0 hr.	6 hrs.	Per cent destruction	0 hr.	6 hrs.	Per cent destruction	0 hr.	6 hrs.	Per cent destruction	0 hr.	6 hrs.	Per cent destruction
Phosphate (M/15).....	41.5	37.0	10.8	39.4	23.9	39.5	39.6	32.6	17.5	39.3	28.6	27.0
Arsenate (M/15).....				40.9	21.4	47.5						
Carbonate-phosphate.....							41.2*	37.0	10.0			
Carbonate (M/15).....							38.8†	38.2	1.5			
Acetate (0.2 M).....	39.3‡	39.5	0	40.0§	38.4	2.6						
Borate (0.2 M).....				38.5	37.7	2.1				41.7	41.9	0
None 	39.8	40.4	0									

* Oxygen tension 70 per cent that in the other experiments.

† pH varied between 7.5 and 8.0 The oxygen tension was 70 per cent that in the other experiments.

‡ The temperature was 88°.

§ pH varied between 7.0 and 7.2.

|| pH varied between 5.8 and 6.5 (unbuffered solution).

phosphate and arsenate upon the destruction of fructose is of interest in the light of the importance of hexosephosphate esters in the metabolism of carbohydrates (5), of the observations of Harden and Young (6) on the accelerating effect of arsenates, as well as phosphates, on the destruction of glucose by yeast juice, and the more recent work of Courtois (7) on the similar behavior of phosphates and arsenates on the action of the vegetable phosphatases. Attempts at isolating a hexosephosphate ester were unsuccessful.

Changes in the concentration of fructose initially present produced but slight alterations in the percentage destruction, while variations in the phosphate concentration caused a marked, nearly proportional shift in the percentage destruction. In $m/30$ phosphate 10.5 per cent of the fructose was destroyed in 6 hours. Doubling this phosphate concentration ($m/15$) increased the destruction to 39.5 per cent. Doubling this new concentration (7.8 m phosphate) increased the percentage destruction to 61.8 per cent. In all these experiments all other factors (pH, temperature, oxygen pressure, initial fructose concentration) were identical. This shows that the amount of phosphate present is a controlling factor in regulating the amount of oxidation. In order to exclude the possibility that the concentration of salt present was affecting the rate of oxidation, an experiment was carried out with pure sodium sulfate as well as $m/15$ phosphate. The results of this experiment showed 37.5 per cent destruction, while phosphate alone under the same conditions showed 39.5 per cent destruction.

Certain previous work reporting the oxidation of hexosephosphate esters in phosphate buffers (8) and the catalyzing action of sodium ferropyrophosphate on the oxidation of sugars in the presence of disodium phosphate (9) suggested that the effect of the phosphate on the destruction of fructose might be due to the presence of metallic salts. Therefore, it seemed advisable to repurify the phosphate salts used in making the buffer solutions. A phosphate buffer solution of pH 7.0 made from twice recrystallized disodium hydrogen phosphate and potassium dihydrogen phosphate showed only 9.7 per cent destruction of fructose under conditions that had shown 39.5 per cent destruction with the stock buffer mixture. This great reduction in destruction suggested that the active agent catalyzing the reaction could be recovered

in more concentrated form from the supernatant liquor of the recrystallized phosphate. A buffer mixture (pH 7.0) of proper strength prepared from this supernatant liquor, however, showed only 12.2 per cent destruction under conditions that had previously shown 39.5 per cent. It was concluded that the destruction of fructose by oxygen in the presence of phosphates may be catalyzed by certain universally occurring impurities. Attempts to concentrate this factor were unsuccessful. The original phosphates used were of the highest quality, fresh samples of Merck's analytical (Blue Label) reagents.

SUMMARY

The interconversion of dilute buffered carbohydrate solutions and the oxidation of fructose by oxygen gas are independent phenomena separable from one another under carefully controlled conditions. The oxidation takes place in the presence of phosphate and arsenate solutions, but not in the other buffer systems studied. The oxidation may be brought about by the catalytic action of traces of impurities in the phosphate and arsenate used. Glucose, treated similarly, is not destroyed under conditions which show up to 40 per cent destruction of fructose in 6 hours.

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GRAVIMETRIC METHOD FOR THE DETERMINATION OF SODIUM PREGNANDIOL GLUCURONIDATE (AN EXCRETION PRODUCT OF PROGESTERONE)

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The isolation and identification of sodium pregnandiol glucuronidate from human urine during pregnancy has been described by Venning and Browne (1) in an earlier communication. Later, Venning, Henry, and Browne (2) reported that, not only is this substance excreted in increasing amounts throughout pregnancy, but it also appears in normal urine during the luteal phase of the menstrual cycle. The very close time relationship between the formation of a corpus luteum in the ovary and the appearance of this compound in the urine, together with the fact that after intramuscular injections of progesterone sodium pregnandiol glucuronidate is excreted in the urine, led the authors to adopt the view that this compound is actually an excretion product of progesterone. Up to the present the assay of progesterone in the urine, blood, or tissues has yielded no information as to the requirements of the body for this hormone. The reduction of progesterone to the biologically inactive compound pregnandiol in the process of metabolism, and the subsequent formation of a water-soluble glucuronide, is a possible explanation of the previous failure to demonstrate the presence of this hormone in any quantity in the body or in the excreta. The following relatively simple gravimetric method of measuring an excretion product derived from progesterone opens up a new field of research with regard to the production of this hormone in the body, both in the pregnant and non-pregnant state. The method has not been extended to blood or tissue but it seems possible that with some modification it might be applied to them.

Method

A 24 hour specimen of urine is collected, measured, and an aliquot amount is taken which may be expected to contain 20 to 40 mg. of the combined pregnandiol. The best results are obtained if the urine is extracted without previous addition of either acid or alkali. The urine is extracted four times with a total of about one-third of its volume of normal butyl alcohol in a separatory funnel. (If a liter of urine has been taken, the volumes of butyl alcohol are 200, 75, 50, and 50 cc.) The combined butyl alcohol extracts are centrifuged or allowed to stand until clear. The supernatant butyl alcohol is poured off into a distilling flask, the precipitate is washed once with butyl alcohol, and the washings are added to the flask. The butyl alcohol is evaporated to dryness under reduced pressure and the residue is taken up in 60 cc. of 0.1 N NaOH. This mixture is again extracted four times with butyl alcohol (20, 20, 10, and 10 cc.). The butyl alcohol is washed twice with 5 cc. of water. At this stage it is almost colorless. It is then centrifuged and the clear butyl alcohol is evaporated to dryness in a 1 liter distilling flask under reduced pressure. Exactly 5 cc. of water are added to the flask. It is warmed on a water bath to about 50° and about 10 cc. of acetone are added. The residue is completely dissolved and the contents are then transferred to a 125 cc. Erlenmeyer flask. The original flask is washed out several times with acetone and the final volume of the mixture is made up to 100 cc. with acetone. It is allowed to stand overnight in a refrigerator at 5-10°. A white precipitate settles out. Most of the supernatant fluid can then be drawn off by suction; the remainder is transferred to a 50 cc. centrifuge tube, and, after it is centrifuged, the acetone is poured off without disturbing the precipitate.

A few drops of water are added to the centrifuge tube and it is warmed on a water bath. The original Erlenmeyer flask is then washed out with hot 95 per cent ethyl alcohol, which is added to the centrifuge tube to dissolve the contents. The hot alcohol solution is filtered with suction into a weighed beaker, evaporated to dryness on a water bath, and the contents are weighed. The purity of the precipitate at this stage may be judged by its color and melting point. Pure sodium pregnandiol glucuronidate melts at 268-271° with decomposition and evolution of gas.

The first precipitate always contains from 10 to 30 per cent of impurities, depending upon the volume of urine and the amount of combined pregnandiol extracted. If a large volume of urine has to be used in order to obtain a measurable yield of the compound, the impurities contained in the precipitate are relatively greater. If truly quantitative values are desired, a second precipitation with acetone and water must always be made. When two precipitations are made, the first residue obtained on addition of acetone is simply redissolved in water and acetone is added. The amount of water to be used is discussed later. This added procedure involves a certain fixed loss of the combined pregnandiol and experiments have been carried out in order to determine the amount of this loss under varying conditions. Certain specimens of urine are found to contain a gummy material which leads to difficulty in the process of extraction. This substance is frequently adsorbed to the precipitate and carried down with it and in consequence the residue is dark brown in color. In order to be certain whether this gummy residue actually contains any combined pregnandiol, it must be redissolved in 0.1 N NaOH, reextracted with butyl alcohol, and the process repeated from that stage on. If the urine contains blood, it is almost impossible to obtain a pure precipitate even when two extractions from 0.1 N NaOH are made.

EXPERIMENTAL

A series of experiments has been performed in order to measure the recovery of added sodium pregnandiol glucuronide from acetone and water, 0.1 N NaOH, and urine.

Recovery from Acetone—Weighed amounts of pure sodium pregnandiol glucuronide were dissolved in varying amounts of water; acetone was added to make the final volume up to 100 cc. The amount of the compound recovered was measured. The results are shown in Table I.

As is seen from the values in Table I, the amount of sodium pregnandiol glucuronide recovered is dependent on the amount of water used in the precipitation. When recovering small amounts of the compound, it is important to reduce the amount of water used to dissolve the residue, particularly in the second

precipitation. The presence of a certain amount of water is necessary, however, in order to keep the impurities in solution.

Recovery from 0.1 N NaOH—Weighed amounts of sodium pregnanediol glucuronide were dissolved in 50 cc. of 0.1 N NaOH, extracted four times with butyl alcohol, washed twice with 5 cc. of water, evaporated to dryness, and precipitated with acetone, 5 cc. of water being used. In order to determine the effects of (1) a second precipitation with acetone and (2) a second extraction

TABLE I

Recovery of Sodium Pregnanediol Glucuronide from Acetone and Water

Na pregnan- diol glucuro- nide	3 cc. water		5 cc. water		10 cc. water	
	mg.	per cent	mg.	per cent	mg.	per cent
3.0	2.0	67	0.8	27	0	0
5.0	3.7	74	3.0	60	1.4	28
10.0	8.1	81	7.6	76	6.0	60
20.0			16.6	83	15.2	76

TABLE II

Recovery of Sodium Pregnanediol Glucuronide from 0.1 N NaOH

Na pregnan- diol glucuro- nide	1st precipitation		2nd precipitation			
			1st recovery		2nd recovery	
	mg.	per cent	mg.	per cent	mg.	per cent
5	3.2	64				
10	8.0	80	6.4	64	7.4	74
20	17.4	87	13.6	68	16.0	80
40	36.0	90	30.4	76	32.0	80
70	64.3	92	61.0	87	62.2	89

with 0.1 N NaOH followed by a second precipitation with acetone on the recovery, two sets of experiments were carried through. After the precipitates were weighed (1) the residues in one of the series were redissolved in 5 cc. of water, acetone was added, and the amount of compound measured; (2) the residues of the second series were redissolved in 0.1 N NaOH and reextracted with butyl alcohol and again precipitated with acetone with 5 cc. of water. The results are shown in Table II.

From a comparison of the first and second recoveries it is appar-

ent that greater amounts of the compound are recovered when it is extracted from 0.1 N NaOH with butyl alcohol and precipitated with acetone than when it is directly dissolved in water and precipitated out. Possibly the small traces of NaOH carried through in the process may decrease the solubility of the compound.

Recovery from Urine—A series of three experiments was carried out on urine. Varying amounts of the compound were dissolved in water and added to a mixed sample of urine containing none of the compound. The process was carried out as described in the section "Method."

TABLE III
Recovery of Sodium Pregnandiol Glucuronide from Urine

Experiment No.	Added Na pregnandiol glucuronide	1st precipitation		Impurity	2nd precipitation	
	mg.	mg.	per cent	mg.	mg.	per cent
1	0	2.2		2.2		
	5	7.5	150	2.5		
	10	12.5	125	2.5		
	15	17.6	117	2.6		
	20	22.8	114	2.8		
	30	33.0	110	3.0		
2	0	6.2		6.2	0	
	5	11.0	220	6.0	1.0	20
	10	17.4	174	7.4	7.0	70
	20	26.4	132	6.4	17.1	85
	40	45.3	113	5.3	34.8	87
3	0	3.2		3.2	0.2	
	10	13.8	138	3.8	8.1	81
	20	23.6	118	3.6	16.8	84
	40	43.8	110	3.8	34.4	86
	70	69.3	99		62.0	88.5

Experiment 1—1000 cc. of urine were used in each case. The process was carried out as far as the first precipitation with acetone. The residues were weighed at this stage.

Experiment 2—1700 cc. of urine were used. This time the first precipitate was redissolved in 5 cc. of water and reprecipitated directly with acetone.

Experiment 3—900 cc. of another lot of urine were used in each case. After the material was weighed following the first precipita-

tion it was redissolved in 0.1 N NaOH, extracted with butyl alcohol, and again precipitated with acetone, 5 cc. of water being used. The results of all three groups are shown in Table III. The weight of the impurity is obtained by subtracting the weight of the sodium pregnandiol glucuronidate added to the urine from the weight of the first precipitate.

DISCUSSION

Sodium pregnandiol glucuronidate is extremely soluble in butyl alcohol and from experiments carried out we have found it to be completely extracted by means of this solvent from an aqueous solution, whether of acid, alkaline, or neutral reaction. However, when this compound is extracted from urine, it is better not to add either acid or alkali for the first extraction with butyl alcohol. If acid is added, large amounts of impurities go into the butyl alcohol, which are difficult to eliminate in the second stage of the process. The same criticism applies to the addition of alkali to the urine. This compound appears to be stable in urine. Three similar specimens of urine were taken: the first specimen was extracted when fresh, the second was allowed to stand in the refrigerator for 4 days, and the third was allowed to remain at room temperature for a similar period. The amounts recovered were 16.9 mg., 16.9 mg., and 16.6 mg. respectively.

From the results obtained in Table III it is shown that the compound is completely extracted from the urine by means of the method outlined and that there is practically no loss of this substance even when the latter is precipitated by means of water and acetone. This may be explained by the assumption that the impurities present in the urinary extract aid complete precipitation of the compound. Another possibility is that the amount which would ordinarily remain in solution in a mixture of acetone and water is adsorbed to the impurities and carried out of solution with them. Whatever may be the explanation it seems to be borne out by experiment that the first precipitation from urinary extracts represents the total amount of compound in the urine with an added amount of impurity. The constancy of the weight of the impurity justifies the assumption that the amount of impurity in the precipitate depends on the sample of urine and does not vary with the amount of compound present. If the volume of

urine extracted is large in comparison to the amount of compound extracted, the presence of the latter may be completely obscured. When a second precipitation is carried out, the correspondingly smaller amount of impurity remains in solution and the precipitate obtained shows a high degree of purity (m.p. 268°). This second precipitation involves a certain loss of the compound which can, however, be calculated. The loss entailed by this second precipitation appears to be of the same order as that occurring when the compound is extracted from 0.1 N NaOH and only one precipitation is carried out.

The advisability of using 5 cc. or 3 cc. of water for the second precipitation depends upon the amount of compound present. When the compound is of the order of 15 to 40 mg., 5 cc. of water are used, when it is lower than 15 mg., 3 cc. of water should be used.

The recovery of the compound is fairly constant for values between 20 and 40 mg., and 85 per cent of the original amount can be recovered when 5 cc. of water are used. As the amount of the compound decreases, the recovery is proportionately less.

The method outlined is accurate for values between 15 and 40 mg. If the urine is expected to contain a large amount of the compound, it is better to reduce the volume extracted. On the other hand, when only 3 to 10 mg. are being excreted, it is advisable to collect 48 hour specimens in order to obtain sufficient compound. With amounts below 5 mg. the recovery is unreliable. However, at this level the method may be used as a qualitative test for the presence or absence of this compound.

Calculation

The percentage recovery of sodium pregnandiol glucuronidate from urine increases with the amount of compound present. With 3 cc. of water in the second precipitation, a final precipitate of 2 to 4 mg. represents 70 per cent of the original compound, 5 to 10 mg. 80 per cent, and 11 to 15 mg. 85 per cent. When 5 cc. of water are used a precipitate from 9 to 12 mg. represents 75 per cent of the original compound, 13 to 18 mg. 82 per cent, 19 to 25 mg. 85 per cent, and 26 to 50 mg. 87 per cent. This must be allowed for when the final calculation is made.

The calculated amount of sodium pregnandiol glucuronidate is converted into terms of pregnandiol excreted per 24 hours. The

molecular weights of pregnandiol and progesterone are 320 and 315 respectively. The excreted amount of pregnandiol may thus be conveniently compared with the corresponding amount of progesterone from which it is considered to be derived.

The formula for sodium pregnandiol glucuronidate is $C_{27}H_{48}O_8 \cdot NaH_2O$; it contains 1 molecule of water of crystallization and has a molecular weight of 536. The factor for its conversion into pregnandiol is $320/536 = 0.597$. After the sodium pregnandiol glucuronidate is weighed, its corresponding original value is determined from the recovery values, and the total amount of pregnandiol excreted per 24 hours is calculated.

Example—800 cc. of a 24 hour specimen of urine of 1500 cc. volume were extracted. Two precipitations with water and acetone were carried out; 5 cc. of water were used in each case and the weight of the residue was 25 mg. Since 25 mg. represent 85 per cent recovery, the original amount in this case was 29.4 mg.

$$\text{Sodium pregnandiol glucuronidate excreted} = \frac{29.4 \times 1500}{800} \text{ mg. per 24 hrs.}$$

$$\text{Pregnandiol} = \frac{29.4 \times 1500 \times 0.597}{800} \text{ mg. per 24 hrs.}$$

Values for the normal excretion of this substance during the menstrual cycle and throughout pregnancy will be published shortly.

SUMMARY

A gravimetric method for determining sodium pregnandiol glucuronidate in urine is described. This compound is considered to be an excretion product of progesterone.

The author wishes to express her appreciation for the technical assistance rendered by Mr. V. Kazmin.

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THE DETERMINATION OF BILIRUBIN WITH THE PHOTOELECTRIC COLORIMETER

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The main source of error in the determination of bilirubin in serum by the diazo reaction has been the loss caused by adsorption on the protein precipitate. In addition, the lack of an accurately matching artificial standard and the sensitivity of the azobilirubin color to changes in pH have made accurate colorimetric determinations impossible, without the aid of some type of objective photometer such as the spectrophotometer, the Pulfrich photometer, or the photoelectric colorimeter (1-4).

In this paper we shall describe a method for the quantitative determination of both direct and indirect bilirubin in serum, in which protein precipitation and consequent loss of bilirubin have been eliminated. By a slight modification of the method, a quantitative study of the behavior of the direct reaction has also been made possible. Artificial standards have been eliminated in the colorimetric determinations by the use of the photoelectric colorimeter (5) with a specially selected light filter.

Selection of Color Filter

The spectrophotometric curve of the rose-mauve color of the azobilirubin solutions obtained by the method to be described below has a single broad absorption band at $540\text{ m}\mu$ (Fig. 1). We have therefore chosen a filter which transmits a narrow spectral band in the vicinity of $540\text{ m}\mu$, so that light which has passed through the filter is readily absorbed by solutions of azobilirubin. This filter has the further advantage of being unaffected by the

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presence of the yellow serum pigments whose absorption at $540\text{ m}\mu$ is negligible. The only other interfering color is that due to the possible presence of hemoglobin in the serum. Although this pigment does absorb light in the vicinity of $540\text{ m}\mu$, any error

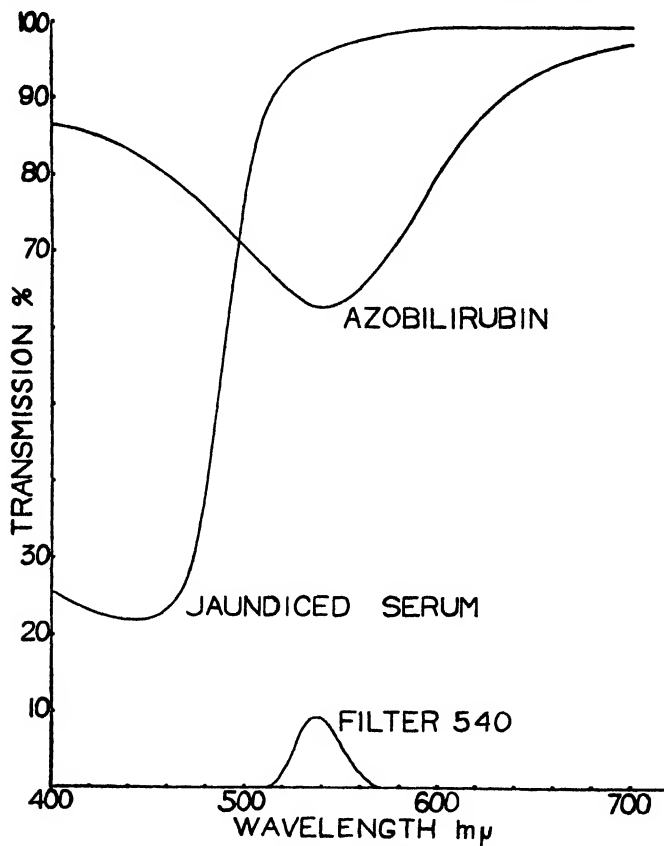


FIG. 1. Spectrophotometric curves of azobilirubin, Filter 540, and jaundiced serum.

from this source is obviated by the use of a blank tube in the initial adjustment of the instrument.

Indirect Reaction

Van den Bergh (6) assumed that indirect bilirubin in serum would only react with diazo reagent in the presence of alcohol,

after the proteins had been removed by precipitation. Our experiments, however, have proved that complete coupling of indirect bilirubin will take place in the presence of serum proteins provided the alcohol concentration is of the order of 50 per cent. This concentration can be achieved without protein precipitation if the serum is first diluted with water, and this procedure forms the basis for our technique for indirect bilirubin. This not only eliminates loss of bilirubin on the protein precipitate, but also provides a buffer substrate sufficient to stabilize the pH-sensitive color of the azobilirubin.

Method

Reagents—

1. Solution A, 1.0 gm. of sulfanilic acid dissolved in 15 cc. of concentrated HCl, and diluted to 1 liter with water.
2. Solution B, 0.5 per cent sodium nitrite.
3. Diazo reagent freshly prepared by adding 0.3 cc. of Solution B to 10 cc. of Solution A.
4. Hydrochloric acid for blank tubes (diazo blank), 15 cc. of concentrated HCl in 1 liter of water.
5. Absolute methyl alcohol. The use of absolute methyl alcohol is recommended, since it yields clearer solutions than 95 per cent ethyl alcohol. The amount of color produced is, however, unaltered.

*Procedure—*Two colorimeter tubes are set up as follows: Tube 1, indirect blank, 5 cc. of absolute methyl alcohol and 1 cc. of diazo blank solution; Tube 2, indirect sample, 5 cc. of absolute methyl alcohol and 1 cc. of diazo reagent.

1 cc. of serum or plasma is diluted to 10 cc. with distilled water, and 4 cc. of the diluted material are added to each tube. The contents are mixed by inversion, care being taken to handle both tubes in the same way, so that any turbidity which may result from too vigorous shaking will be the same in both tubes. If bubbles form they are best removed by gentle tilting and rotation of the tubes.

Tube 2 is read in the colorimeter with Filter 540¹ 30 minutes

¹ This filter is one of the set of eight filters which have been selected for use with the photoelectric colorimeter. Any of these filters may be obtained from the Rubicon Company, 29 North 6th Street, Philadelphia. The complete colorimeter may also be obtained from the Rubicon Company.

after addition of the serum, Tube 1 being used for the initial adjustment of the galvanometer. If the galvanometer reading is less than 10, it is advisable for the sake of greater accuracy to dilute both tubes with 10 cc. of 50 per cent methyl alcohol and read again immediately. In this case, the final answer in mg. per 100 cc. must be multiplied by 2.

Calculation—The bilirubin concentration in mg. per 100 cc. of serum is obtained from the formula

$$X = \frac{2 - \log G}{6.72} \times 100$$

where X is mg. of bilirubin per 100 cc. of serum, and G is the galvanometer reading.

For routine determinations, a calibration curve may be made from this formula from which the values for X may be read directly (Fig. 2).

Calibration—Since the concentration of azobilirubin in a solution is proportional to the negative logarithm of the light transmission, the following formula is valid

$$C = \frac{2 - \log G}{K_1} \quad (1)$$

where C is the concentration of azobilirubin (expressed as bilirubin) in mg. per cc. of colored solution, G is the galvanometer reading, and K_1 is a constant.

Conversion of the bilirubin concentration in terms of mg. per cc. of colored solution to mg. per 100 cc. of serum is made by means of the formula

$$X = \frac{2 - \log G}{K_1} \times \frac{V}{A} \times 100 \quad (2)$$

where X is mg. of bilirubin per 100 cc. of serum, V is the volume of colored solution, and A is the amount of serum used.

Thus in the method described above

$$X = \frac{2 - \log G}{K_1} \times \frac{10}{0.4} \times 100 \quad (3)$$

Calibration of the instrument consists essentially in determining the value of K_1 by obtaining the galvanometer readings for known

concentrations of bilirubin. For this purpose about 10 mg. (accurately weighed) of pure bilirubin² were dissolved in 100 cc. of chloroform. Portions were withdrawn from this solution and diluted with ethyl alcohol to a final concentration of 0.01 mg. per cc. Varying amounts of the alcoholic solutions were placed in a series of colorimeter tubes to which were added 1 cc. of diazo reagent and sufficient ethyl alcohol to make the final volume 10 cc. The tubes were read at 5 minute intervals until the color was at a maximum. The known values for C and G were then substituted

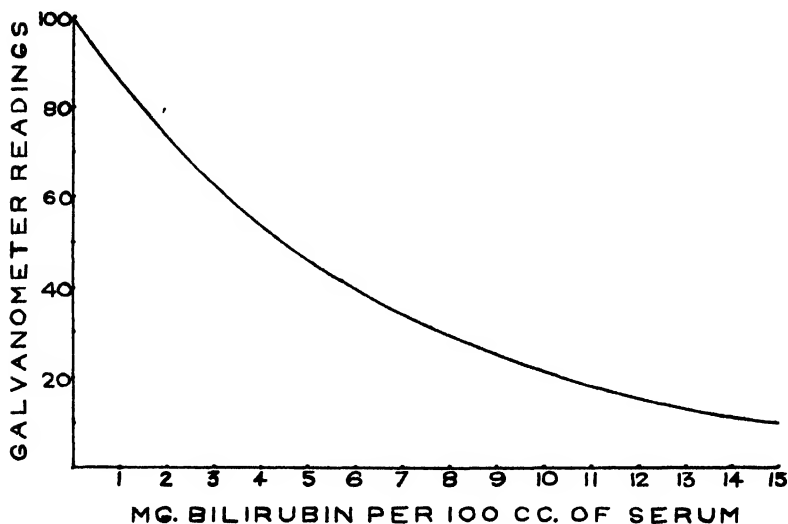


FIG. 2. Calibration curve. This curve has been checked at frequent intervals for nearly 2 years, and no variation greater than ± 1 per cent has been found.

in Equation 1, and the value of K_1 was found to be 168.0. Since all photoelectric colorimeters of this type are interchangeable with respect to calibration, we recommend that this value of K_1 be generally adopted to insure uniformity of results.

² We have tested both Eastman Kodak and Hoffmann-La Roche bilirubin, but have used the latter for calibration, since the yield of color is 10 per cent higher. Moreover, van den Bergh and Grotepass (7) state that they have found Hoffmann-La Roche bilirubin to be identical with the chemically pure bilirubin prepared by Professor Hans Fischer.

Substituting K_1 in Equation 3 above we obtain

$$X = \frac{2 - \log G}{6.72} \times 100 \text{ mg. bilirubin per 100 cc. serum}$$

The value 6.72 we have termed K_2 .

Results

Effect of Alcohol Concentration upon Color Development—The curve in Fig. 3 marked 0 per cent shows the color produced when

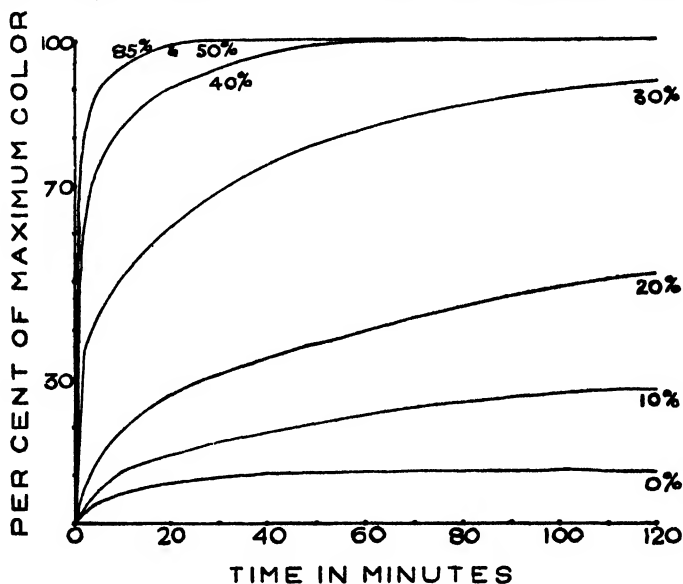


FIG. 3. Effect of alcohol concentration on development of color from bilirubin.

diazo reagent, without alcohol, is added to serum. By definition, this must be due to direct bilirubin only. The addition of alcohol in increasing amounts causes more and more of the indirect bilirubin to take part in the reaction, until at concentrations above 40 per cent the indirect reaction is complete. An alcohol concentration of 50 per cent was chosen for the method, since it affords an adequate margin of safety, yields perfectly clear solutions, and produces maximum color in a shorter time than 40 per cent.

Recovery of Bilirubin Added to Serum—Table I shows that bilirubin added to serum is recovered with a maximum error of

TABLE I

Recovery of Pure Indirect Bilirubin Added to Normal and Jaundiced Sera

0.4 cc. of serum was used in each case. The figures in the second column represent the amount of bilirubin present in this 0.4 cc., as measured directly on the instrument. The first five experiments were made on normal sera. (Normal values obtained by our method are between 0.2 and 0.8 mg. per 100 cc.)

Bilirubin per 100 cc. serum	Bilirubin in 0.4 cc. serum	Bilirubin added to 0.4 cc. serum	Total bilirubin present	Amount of bilirubin measured	Percentage recovery
mg.	mg. $\times 10^{-3}$	mg. $\times 10^{-3}$	mg. $\times 10^{-3}$	mg. $\times 10^{-3}$	
0.25	1.0	4.4	5.4	5.5	101.8
0.5	2.0	8.8	10.8	10.6	98.1
0.5	2.0	50.0	52.0	50.0	96.1
0.75	3.0	34.0	37.0	36.4	97.7
0.75	3.0	80.0	83.0	80.0	96.3
1.0	4.0	43.6	47.6	48.0	100.8
1.0	4.0	88.0	92.0	95.0	103.4
1.7	6.8	31.2	38.0	38.4	101.1
2.7	10.8	30.0	40.8	40.0	98.0
3.2	12.8	50.0	62.8	63.4	101.0
4.0	16.0	40.0	56.0	54.0	96.5
4.0	16.0	50.0	66.0	67.4	102.0
8.0	32.0	40.0	72.0	73.2	101.9
16.1	64.0	20.0	84.0	83.6	99.7

TABLE II

Comparison of Determinations Made on Same Sera by Different Techniques

All the figures represent mg. per 100 cc. of serum. All the final colorimetric measurements were made on the photoelectric colorimeter.

Technique	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
Van den Bergh and Grotepass (7) (indirect reaction).....	2.0	4.2	3.1	7.8	12.4
Thannhauser and Andersen (8)....	3.3	4.9	4.6	9.7	12.8
Jendrassik and Czike (9).....	3.4	4.9	5.2	10.5	15.1
Malloy and Evelyn (indirect reaction).....	4.8	6.5	9.0	15.2	22.8

± 4 per cent, the average error being only 2 per cent. Since the calibration curve used in these experiments was made with known

amounts of pure indirect bilirubin in alcoholic solution, the results prove that the presence of serum proteins does not interfere with the diazo reaction of bilirubin, or with the quantitative determination of the resulting azobilirubin by means of the photoelectric colorimeter.

Duplicate determinations on serum will usually agree within ± 1 per cent, with a maximum variation of ± 2 per cent.

Comparison with Other Methods—The results of duplicate analyses on the same sera by different methods are shown in Table II. All the final colorimetric measurements were made on the photoelectric colorimeter so that any discrepancies which occurred could only have been due to differences in the preliminary treatment of the serum. From Table II we conclude that (1) precipitation of proteins before addition of the diazo reagent, as recommended by van den Bergh and Grotepass (7), causes a large and variable loss of bilirubin (30 to 60 per cent); (2) this loss can be decreased but not eliminated by the technique of Thannhauser and Andersen (8), in which the diazo reagent is added before the proteins are precipitated; (3) the addition of caffeine sodium benzoate, as suggested by Jendrassik and Czike (9), is a further slight improvement, but the recovery of azobilirubin is still seldom more than 70 per cent, as compared with our method.

Direct Reaction

Van den Bergh and Grotepass (7), have recommended a quantitative method for the direct reaction, but, since 25 per cent alcohol is used, the method measures not only direct bilirubin but also a fraction of the indirect (see Fig. 3). The results obtained are therefore too high, and since the amount of direct bilirubin in any serum bears no constant relationship to the amount of indirect bilirubin, the error involved is variable.

For routine determinations in which a clinical interpretation is required, we recommend that the direct reaction be carried out in the accustomed manner. For a more quantitative study of the behavior of the direct reaction, we have, however, adopted the following procedure: Two tubes are set up as in the method for the indirect reaction except that 5 cc. of water are substituted for 5 cc. of methyl alcohol. Readings are made on the photoelectric colorimeter at 10, 30, 60, and 120 minutes, and the corre-

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sponding bilirubin concentrations, obtained from the calibration curve, are plotted to show development of color with time.

Curve C of Fig. 4 shows the slow development of color from a serum of the "delayed" type, in which the true end-point of the reaction is not attained for several hours. Curve A shows the rapid development of maximum color typical of sera of the "prompt" type. Curve B shows a reaction intermediate between these extremes, which corresponds to what is usually termed the "biphasic" reaction.

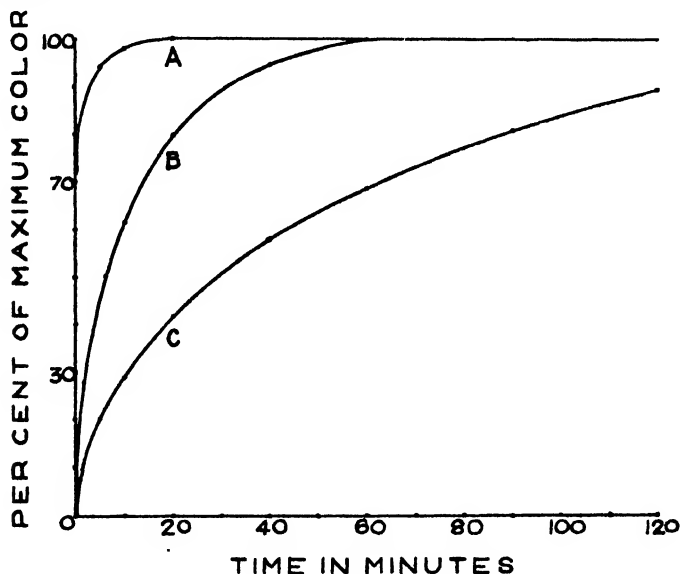


FIG. 4. Behavior of the direct reaction in different sera; Curve A "prompt," Curve B "biphasic," and Curve C "delayed."

In addition, the final galvanometer reading is used to determine the concentration of direct bilirubin in the serum. Sera have been found to differ not only in the shape of their color development curves, but also in the ratio of the amounts of direct and indirect bilirubin. The clinical significance of these two variables will be discussed elsewhere.

SUMMARY

1. A method has been described for the accurate photoelectric determination of both direct and indirect bilirubin in serum, in

which protein precipitation and consequent loss of bilirubin by adsorption have been eliminated.

2. The interfering effect of yellow serum pigments in the color determination has been overcome by the use of a specially selected light filter, which also eliminates the necessity for artificial color standards.

3. It has been shown that all the bilirubin in serum will react with diazo reagent even in the presence of serum proteins, provided a sufficiently high concentration of alcohol (50 per cent) is present.

4. The new method has been found to give higher and consistently more accurate results than any of the older methods with which it has been compared. Bilirubin added to serum is recovered with an average error of ± 2 per cent.

5. By a modification of the method a quantitative study of the behavior of the direct reaction of bilirubin in serum has been made possible.

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ON GLYCOPROTEINS

IV. THE ESTIMATION OF HEXOSAMINE

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The first practical method for the estimation of hexosamine was that of Elson and Morgan (1), in which the reaction of Pauly and Ludwig (2) is used. The method published earlier by Zuckerkandl and Messiner-Klebermass (3), embodying the same general reactions, has been useful in the hands of only a few (4). Since its publication, the Elson and Morgan method has been used and modified by a number of investigators (5-8), because the estimation of hexosamine in the presence of large amounts of protein did not appear feasible (9). The most successful of these investigators is Nilsson (8) who has very recently described a procedure by means of which glucosamine hydrochloride added to casein and serum can be estimated with an accuracy of 90 to 108 per cent.

In a series of studies on the sugar groups in glycoproteins (10-14) we also modified the method, because in our hands the intensity of color developed by hexosamine in the range of concentration used by Elson and Morgan (0.75 to 3.0 mg. per 2 cc. sample) and later by Nilsson (0.08 to 0.24 mg. per 2 cc. sample) did not obey the Beer-Lambert law; this difficulty did not appear over the range of 2.5 to 25 micrograms per 1 cc. sample. Furthermore, there is reason to doubt that the conditions of hydrolysis adopted by Nilsson lead to the complete liberation of hexosamine. In spite of his observation that isolated mucins and mucoids require 12 hours of hydrolysis with N HCl, he employs only 3 hours for blood serum, the value so obtained being 0.6 mg. per cc. Estimation of the combined hexosamine in normal human blood serum by the procedure here reported yields values of 0.9 to 1.3 mg. per cc. The hexosamine content of polysaccharides, estimated by

our method, is in satisfactory agreement with their other constituents. The variation of the results obtained in the presence of large proportions of protein is not more than ± 7 per cent.

Reagents—

1. Approximately 8 N HCl.
2. Approximately 0.25 N NaOH.
3. Acetylacetone solution, prepared immediately before use by dissolving 0.2 cc. of acetylacetone in 10 cc. of 0.5 N Na_2CO_3 . The acetylacetone (Eastman Kodak No. 1088) was kept cold.
4. Aldehyde-free alcohol, prepared from commercial absolute alcohol by the customary treatment with Ag_2O and NaOH followed by redistillation in an all-glass apparatus (15).
5. *p*-Dimethylaminobenzaldehyde. The Eastman Kodak product was freed of a yellow impurity by dissolving in concentrated HCl (16), diluting with water, and fractionally precipitating by the addition of saturated sodium acetate solution. Only the pure white, later fractions are employed for the test; the earlier fractions, which contain the impurity, yield further quantities of colorless material on repeated fractional precipitation.
6. Ehrlich's reagent, prepared by dissolving 0.8 gm. of purified *p*-dimethylaminobenzaldehyde in 30 cc. of aldehyde-free alcohol and adding 30 cc. of concentrated HCl. This solution was kept in the refrigerator.

Procedure

A sample of the substance (to contain 0.25 to 1.25 mg. of hexosamine) is weighed or measured into a 13 \times 100 mm. Pyrex test-tube. Water is added to 1 cc. and 1 cc. of approximately 8 N HCl is added. The tube is sealed off and heated in boiling water for 8 hours. The contents are then transferred to a 25 cc. volumetric flask and made up to volume. 10 cc. are withdrawn and titrated to the turning point of methyl red with 0.25 N NaOH. Another 10 cc. of the diluted hydrolysate are then transferred to a 25 cc. volumetric flask, neutralized with 98 to 99 per cent of the amount of alkali required in the preliminary titration (efficient stirring is necessary during the neutralization), and made up to volume.

An aliquot of the neutralized hydrolysate (1 cc.) or 1 cc.

of a solution containing hexosamine is pipetted into a 5 cc. Pyrex volumetric flask, and 0.5 cc. of freshly prepared acetylacetone solution is added from a 0.5 cc. Ostwald-Folin pipette. The flasks, held in a wire basket, are immersed in boiling water to a depth sufficient to cover the solution; the necks are exposed to a strong current of air. After 15 minutes the flasks are cooled by immersion in tap water. The contents are diluted with 2.5 cc. of aldehyde-free alcohol and 0.5 cc. of Ehrlich's reagent is added with stirring. Alcohol is then added to the mark and the flasks are shaken, care being taken to permit escape of CO_2 . Two standard solutions, containing approximately 7.5 and 15 micrograms of glucosamine per cc., and a blank (1 cc. of water) are treated simultaneously in the same manner. The flasks are then placed in an incubator at 37° for 30 minutes. The light absorption of the test solutions and the standards is compared with that of the blank in a Pulfrich step photometer (Zeiss), Filter S-53 and 50 mm. microcells being used. The amount of hexosamine is calculated from the readings by means of a calibration curve prepared previously with solutions of glucosamine hydrochloride. In case of a disparity between the concentrations of the standard solutions and the values indicated for them by the curve, the correction found necessary for them is applied to all solutions analyzed at the same time. (Such a correction is seldom necessary.) The final colors developed from solutions of glucosamine and chondrosamine hydrochlorides are identical spectrophotometrically and of equal intensity for equal weights of the sugars.

DISCUSSION

Since the method involves two different chemical reactions and is performed with minute quantities of hexosamine, the conditions described should be adhered to in every detail.

Hydrolysis—The hydrolysis of samples of the polyuronic acids of vitreous humor and umbilical cord (13) with 4 N HCl in boiling water for 8 hours gave a maximal value for hexosamine, a value in good agreement with the nitrogen and hexuronic acid determinations and the equivalent weight titrations of the polysaccharides. Hydrolysis with 2 N HCl occasionally gave lower

values. The maximal reducing power of the solutions toward ferricyanide was obtained in 4 hours with 2 N HCl, the hydrolysis leading to simultaneous decarboxylation of the uronic acid.

Neutralization—Neutralization of the hydrolysates with an equivalent amount of 2 N NaOH as previously described often led to erratic results. In view of the very great sensitivity of hexosamine to alkali the use of more dilute alkali is indicated.¹ A slight acidity is permissible in the final hydrolysate, since it does not appreciably change the alkalinity after the addition of the acetylacetone solution. Nilsson (8) has cautioned against alkalization of the hydrolysate, but with his procedure (titration of a solution less than 1 N in acid with 4 N NaOH) overneutralization is easily possible. Evaporation to dryness for removal of HCl (3, 6) was not feasible. Neutralization of the hydrolysates and solution of the standard glucosamine HCl should be done not more than 1 hour before beginning the analysis.

Reaction with Acetylacetone—The Eastman Kodak product was usually used without further purification. With one lot, however, a blank having a color indistinguishable from that due to hexosamine was always obtained, despite purification of the reagent through the copper salt (17) and redistillation.²

The concentration of acetylacetone and time of heating of the alkaline mixture employed by Elson and Morgan (1) were found to be optimal for the amounts of hexosamine in our procedure. All flasks must be heated uniformly and simultaneously; a shallow water bath heated by an electric hot-plate proved satisfactory.

*Reaction with *p*-Dimethylaminobenzaldehyde*—Of considerable influence on the intensity of the color developed was the purity

¹ The reducing power of the solutions was not altered by such overneutralization.

² When water containing a trace of oxidizing agent (Br_2) was used for the preparation of the blank, a color was obtained in the final solution likewise indistinguishable from that due to hexosamine. This suggests that reactions other than the formation of a pyrrole derivative (1, 3, 7) and its coupling with *p*-dimethylaminobenzaldehyde may be involved in the formation of the color. This color formation by oxidizing agents or impure reagents demonstrates the necessity of using a true blank and not a mixture of equal parts of alcohol and concentrated HCl as recommended by Nilsson (8).

of the alcohol used for the dilution of the alkaline mixture and for the preparation of Ehrlich's reagent. With constant amounts of glucosamine, the color was roughly inversely proportional to the amount of aldehyde present in the alcohol as indicated by the fuchsin test (18). The aldehyde was removed by Ag_2O and NaOH (15) and redistillation in a Pyrex still. The use of glacial acetic acid instead of alcohol (7) invariably produced a brilliant purplish red blank.

The yellow impurity present in commercial *p*-dimethylamino-benzaldehyde gave an intense greenish yellow color in the blank and experimental samples. The pure white, purified product yields a colorless solution in alcohol, but after addition of the HCl the solution assumes a pale greenish yellow color. Like that produced by the commercial aldehyde, this color gives no light absorption with Filter S-53 and does not interfere with the determination.

The color produced was found to be constant over a period $\frac{1}{2}$ to 2 hours after the addition of the Ehrlich's reagent; it is appreciably weaker 3 hours after addition. Under the conditions specified, fifteen to twenty-five samples may be read before the color fades significantly.

The calibration curves were made with known amounts of repeatedly recrystallized glucosamine hydrochloride, the purity of which was checked by optical rotation, analysis for nitrogen (Dumas), and formol titration. With freshly prepared Ehrlich's reagent, the molecular extinction coefficient of the color produced was 8030 over the range of 2.5 to 25 micrograms of glucosamine per 5 cc. of final colored solution. The molecular extinction coefficient fell somewhat when the Ehrlich's reagent solution was kept for several weeks, even in the refrigerator.

Contrary to the experience of Boyer and Fürth (7), no difficulty was experienced in determining glucosamine in the presence of casein hydrolysates. Approximately 1.0 and 2.0 mg. quantities of glucosamine hydrochloride were added to 10 mg. samples of casein; after hydrolysis the proper amounts of glucosamine were found in each case. A control sample of casein with no added glucosamine developed no color in the analysis.

Furthermore no color developed with hydrolysates of edestin,

TABLE I
Recovery of Added Hexosamine

Test substance	Added hexosamine*	Total hexosamine		$\frac{\text{Found}}{\text{Calculated}} \times 100$
		Found	Calculated	
	mg.	mg.	mg.	
0.5 cc. serum 1:3	0	0.21		
0.5 " " 1:3	1.26 GlA	1.395	1.47	94.9
0.5 " " 1:3	0	0.16		
0.5 " " 1:3	1.04 GlA	1.17	1.20	97.5
0.5 " " 1:3	2.08 "	2.23	2.24	99.6
0	0.52 "	0.49	0.52	94.3
0	1.04 "	0.97	1.04	93.3
0	2.08 "	2.06	2.08	99.0
1.0 cc. serum 1:5	0	0.207		
1.0 " " 1:5	1.15 GlA	1.384	1.36	101.8
1.0 " " 1:5	0.82 "	1.053	1.03	102.2
1.0 " " 1:5	0.70 "	0.974	0.91	107.0
1.0 " " 1:3	0	0.388		
1.0 " " 1:3	1.07 GlA	1.40	1.46	95.9
0	0.93 "	0.90	0.93	96.8
0.5 cc. serum 1:5	0	0.125		
0.5 " " 1:5	0	0.131		
0.5 " " 1:5	1.24 GlA	1.38	1.37	100.7
0.5 " " 1:5	0.78 "	0.894	0.91	98.3
0.5 " " 1:3	0	0.211		
0.5 " " 1:3	0	0.213		
0.5 " " 1:3	0.93 GlA	1.131	1.14	99.2
0.5 " " 1:3	0.78 "	0.934	0.99	94.4
0	1.38 "	1.400	1.38	101.4
9.27 mg. edestin	0	0		
9.08 " "	1.60 GlA	1.55	1.60	96.9
11.08 " "	0.84 "	0.83	0.84	98.8
0	1.55 "	1.53	1.55	98.7
12.13 mg. edestin	0.78 ChA	0.79	0.78	101.3
9.51 " "	1.50 "	1.50	1.50	100.0
0	1.42 "	1.43	1.42	100.7

TABLE I—*Concluded*

Test substance	Added hex- osamine*	Total hexo- samine		Found Calculated $\times 100$
		Found	Calcu- lated	
	mg.	mg.	mg.	
4.58 mg. chondroitinsulfuric acid	0	1.111		
4.42 mg. chondroitinsulfuric acid	0	1.09		
1.49 mg. chondroitinsulfuric acid	0.97 GlA	1.25	1.33	94.0
2.45 mg. chondroitinsulfuric acid	1.10 "	1.64	1.69	97.4
0.70 mg. chondroitinsulfuric acid	1.18 ChA	1.29	1.35	95.6
0.56 mg. chondroitinsulfuric acid	0.88 "	0.954	1.01	94.5
0	1.32 "	1.23	1.32	93.3
3.06 mg. vitreous polysaccharide	0	1.114		
1.82 " " "	0.80 GlA	1.47	1.46	100.6
0	0.99 "	0.982	0.99	99.2

* GlA = glucosamine, added as the hydrochloride, but calculated as the base; ChA = chondrosamine, added as the hydrochloride, but calculated as the base.

crystalline insulin,³ crystalline trypsin and chymotrypsin,⁴ and glucosaminic acid prepared according to Pringsheim and Ruschmann (19). No influence on the color developed with glucosamine was obtained by addition of glucose, alanine, or ammonium sulfate, with or without glucose.

Recovery Experiments—Table I summarizes the results of experiments in which known amounts of both glucosamine and chondrosamine hydrochlorides (calculated as free bases in Table I) were added before the hydrolysis of the various substances which did or did not contain hexosamine. The control hexosamine solutions were carried through the same procedure. Since

³ For this we are indebted to Dr. O. Wintersteiner of the Department of Biological Chemistry.

⁴ We wish to thank Dr. J. H. Northrop of The Rockefeller Institute for Medical Research, Princeton, for these preparations.

a considerable amount of humin formation takes place in the hydrolysis of serum, the solutions were usually filtered after being transferred into the 25 cc. volumetric flask, and the aliquots for neutralization were taken from the filtrate. The hexosamine content of normal human serum varied between 0.9 and 1.30 mg. per cc.

Table II summarizes the molar ratio of the hexosamine to some other constituent in a number of substances prepared in this

TABLE II
Hexosamine Content in Relation to Other Constituents

Type of material	Hexosamine content*	Reference analysis		Molar ratio between hexosamine and reference substance
		Substance	Con- stant*	
	<i>per cent</i>		<i>per cent</i>	
Umbilical cord polysaccharide (containing no sulfur)	40.2	Hexuronic acid	45.1	0.976
		Nitrogen	3.23	0.974
Vitreous humor polysaccharide	39.3	Hexuronic acid	45.3	0.950
		Nitrogen	3.23	0.952
Gonadotropic hormone of pregnancy urine	7.72	Reducing sugar as glucose (Hagedorn-Jensen) after acid hydrolysis	16.35	0.475
Ovomucoid	12.50	Reducing sugar (as above)	22.90	0.548†
"	10.99	" " " "	21.73	0.508†

* On ash-free basis.

† The finding of a ratio of hexosamine to total reducing power of nearly 0.5 is significant (in the absence of uronic acid) in view of similar results with ovomucoid obtained by Karlberg (20) and Masamune and Hoshino (21). It appears that the finding of a glucosamine dimannoside, in the polysaccharide of ovomucoid by Levene and Mori (22) needs reinvestigating.

laboratory. Further examples may be found in other papers (10, 13, 14, 23).

The method has also been used to ascertain the amount of hexosamine present in normal and pathological aqueous humor, the procedure being modified to allow for the low concentration of hexosamine and the small amount of fluid available for such study. Two samples of aqueous humor from two cases of glaucoma gave values of 17.2 and 13.8 micrograms of hexosamine

per cc. Normal dog aqueous humor gave a figure of 17.8 micrograms of hexosamine per cc. Experiments on this fluid will be reported elsewhere. In a few normal cerebrospinal fluids no hexosamine could be detected, even if much larger volumes of fluid were used for hydrolysis. The method could here be used to test the permeability of the blood capillaries in cerebral diseases.

SUMMARY

The hexosamine method of Elson and Morgan (1), with the modifications described, has been found satisfactory. The most important of the modifications are:

1. A reduction of the amount of hexosamine used in the analysis from 0.35 to 1.5 mg. per cc. to 2.5 to 25 micrograms per cc.; in the latter range the intensity of the color developed is proportional to the amount of hexosamine present.

2. The use of freshly prepared acetylacetone solutions and of specially purified alcohol.

3. The introduction of a period of heating after the addition of the Ehrlich's reagent to hasten and complete the development of the final color.

Conditions are specified for maximal hydrolysis of hexosamine-containing complexes and for preparation of the hydrolysates for analysis.

The results of a few typical analyses are given.

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ON GLYCOPROTEINS

V. PROTEIN COMPLEXES OF CHONDROITINSULFURIC ACID

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The polyuronic acids of vitreous humor and umbilical cord (1) combine with various proteins to form complexes, which in composition and behavior resemble the substances designated as "mucoids" (2-5) obtained from various organs. Chondroitin-sulfuric acid gives similar but more stable complexes.

The complex formation of chondroitinsulfuric acid and gelatin was noted by Mörner (6). The complexes formed from gelatin and sugar acids, among them chondroitinsulfuric acid, were extensively studied from a physicochemical viewpoint by a group of Dutch investigators (for bibliography, see (7)), who termed the phenomenon "complex coacervation," rather than salt formation.

In this paper are described the properties and composition of some protein complexes with chondroitinsulfuric acid, from which it appears that the complexes are true salts formed in stoichiometric proportions by the union of the basic groups of the proteins and the acid groups of the polysaccharide. Chondroitin-sulfuric acid occurs in nature in such complexes, which are present in structurally organized elements of sheet or fiber form; this type of organization appears in some of the complexes prepared *in vitro*.

EXPERIMENTAL

The chondroitinsulfuric acid used was a sodium salt of about 55 per cent purity;¹ a purified product (8) gave similar results. The proteins studied were gelatin (Gold Label), edestin, crystallized egg albumin, and globin from horse hemoglobin.² The com-

¹ We thank Dr. David Klein of The Wilson Laboratories, Chicago, for this material.

² We thank Mr. E. Borek of the Department of Biological Chemistry for this sample.

plexes were formed by mixing a solution containing the protein with a neutral solution of the chondroitinsulfuric acid and adding acetic acid to 2 per cent concentration. Only with globin was a precipitate obtained in neutral solution.*

Egg Albumin—The chondroitinsulfate of egg albumin was prepared by mixing 20 cc. of a solution of thrice recrystallized egg albumin, containing about 1 mg. of N per cc., with 10 cc. of a neutralized 4 per cent solution of chondroitinsulfuric acid. On adding acetic acid to a concentration of 2 per cent, a precipitate formed. This was purified by careful addition of *N* NaOH, centrifugation, and reprecipitation with acetic acid. This process was repeated. The final precipitate had the consistency of a thick syrup. After washing and drying with alcohol and ether, the analytical values⁴ were: ash, 3.1 per cent; N, 12.3 per cent; reducing substance as glucose after hydrolysis (Hagedorn-Jensen, without Zn(OH)_2 precipitation), 9.75 per cent; hexosamine, 4.78 per cent; ratio of hexosamine N to total N, 0.030.

Globin—In a similar experiment with globin prepared according to Troensegaard (11), a precipitate was obtained at neutral reaction in the form of fine threads, which were dissolved by addition of *N* NaOH and reprecipitated by neutralization. After being washed and dried by alcohol and ether, the material had an analysis of ash, 0.8 per cent; N, 13.4 per cent; reducing substance after hydrolysis (Hagedorn-Jensen without Zn(OH)_2 precipitation), 9.18 per cent; hexosamine, 4.68 per cent; ratio of hexosamine N to total N, 0.027.

In the next experiment, the globin-chondroitinsulfate was precipitated from 2 per cent acetic acid solution. 1 gm. of globin was mixed with 1 gm. of neutralized chondroitinsulfuric acid in 200 cc. of water. The solution was brought to 2 per cent acetic acid and allowed to stand in the ice box overnight, centrifuged,

* The polysaccharides of vitreous humor and umbilical cord also formed a precipitate with globin in neutral solution.

⁴ Nitrogen was estimated by the micro-Kjeldahl method, 2 drops of H_2O_2 being used to aid the oxidation. Amino sugar was estimated by our modification (9) of the method of Elson and Morgan (10). Moisture content was estimated by heating 20 mg. samples in small crucibles at 90° to constant weight; ash was determined in the same sample by igniting at bright red heat for 2 to 3 hours in an electric crucible furnace.

and the precipitate washed three times with 2 per cent acetic acid, then with alcohol, and dried with alcohol, acetone, and ether. The material (0.906 gm.) had an analysis of ash, 1.37 per cent; N, 10.39 per cent; hexosamine, 10.82 per cent; ratio of hexosamine N to total N, 0.0814.

Gelatin and Edestin—In the experiments reported in Table I, constant amounts of gelatin and edestin were used with varying amounts of chondroitinsulfuric acid. It will be seen that the weight of precipitate obtained and its ratio of sugar N to total N were constant except at the lowest concentration of the poly-

TABLE I
Complexes Formed with Varying Amounts of Chondroitinsulfuric Acid

Protein, 1 gm.	Chondroitin-sulfuric acid	Final volume	Weight of ppt.	Ash	Nitrogen	Hexosamine	Sugar N Total N
	gm.	cc.	gm.	per cent	per cent	per cent	
Gelatin*	1.00	200	0.94	0.9	12.2	8.5	0.054
"	0.75	150	0.89	0.5	12.2	8.7	0.056
"	0.50	100	0.96	0.8	12.5	8.4	0.053
"	0.25	50	0.66	2.7	12.9	7.3	0.044
Edestin.....	1.00	200	1.27	2.4	12.6	10.8	0.067
"	0.75	150	1.26	0.8	13.1	10.4	0.062
"	0.50	100	1.25	0.7	13.4	9.3	0.054
"	0.25	50	0.90	1.0	13.3	5.3	0.031

All precipitates were allowed to settle on standing in the ice box overnight, centrifuged, washed with 2 per cent acetic acid, and dried with alcohol and ether before analysis.

* This gelatin contained 2.1 per cent of ash.

saccharide. The gelatin was dissolved in water, the edestin in 2 per cent acetic acid. The precipitate obtained with gelatin after acidification settles on standing in the ice box in the form of a coherent elastic mat of considerable tensile strength, resembling the ground substance of bone. On heating it disperses to oily globules which, on cooling, coalesce again to the rubbery consistency. The material under pressure shows double refraction in polarized light. It is insoluble in water and weak alkali, swells in 0.5 N HCl, and dissolves in stronger acid.

With edestin a coherent stringy elastic mass is obtained on stirring, showing under the microscope a matted appearance, not unlike elastic fibers. These fibers are doubly refractive.

In the next experiment (Table II), the volume of the solution and the carbohydrate concentration were kept constant, while the protein concentration was varied. The first two samples formed lumps of rubbery material which could not be washed free of excess reactants. Table II shows an amount of precipitate roughly proportionate to the amount of gelatin (Samples 2 to 6) within a range of ± 40 mg. in the weight of the precipitate. The preparations were almost ash-free and the ratio of sugar N to total N was constant.

TABLE II

Complexes Formed with Varying Amounts of Gelatin

Chondroitinsulfuric acid, 1.00 gm.; final volume, 200 cc.

Sample No.	Gelatin*	Weight of ppt.	Ash	Nitrogen	Hexosamine	Sugar N Total N
	gm.	gm.	per cent	per cent	per cent	
1	5.00	5.75	0.2	12.8	5.5	0.034
2	3.00	3.10	0.5	11.7	6.2	0.041
3	1.00	1.18	0.5	11.9	7.6	0.050
4	0.75	0.91	0.9	12.1	7.8	0.050
5	0.50	0.65	0	12.4	8.0	0.050
6	0.25	0.37	1.1	12.4	8.0	0.050

All precipitates were allowed to settle on standing in the ice box, centrifuged, washed with 2 per cent acetic acid, and dried with alcohol and ether before analysis.

* This gelatin contained 2.1 per cent of ash.

DISCUSSION

The complexes of chondroitinsulfuric acid with different proteins vary in their hexosamine content and in their physical properties. However, the complex obtained from the polysaccharide and any single protein displays a remarkably constant composition, in spite of quite wide variation in the relative amounts of the two reactants used. This suggests that the precipitate is a true salt of chondroitinsulfuric acid in stoichiometric relation with the basic groups of the protein.

To test further this idea, the acid-binding capacities of the different proteins were calculated (in milli-equivalents per gm.) from the hexosamine contents of the complexes. The findings are shown in Table III together with comparable data from the litera-

ture on binding capacities for acid dyes (12) and on basic amino acid content (13, 14). The agreement is satisfactory with the exception of the globin complex. However, our figure of 1.686 corresponds to an acid equivalent of 1.48 (15) or 1.59 (16) for hemoglobin, determined electrometrically. The acid equivalent of globin of 1.38, determined by the same method (15), seems too low in comparison, since globin should have a greater acid-binding capacity than hemoglobin.⁵ From this we conclude: (1) The chondroitinsulfuric acid complexes are salts like the dye-protein complexes. (2) Chondroitinsulfuric acid reacts with the proteins as a dibasic acid; *i.e.*, both the sulfuric acid group and

TABLE III
Comparison of Acid-Binding Capacity

Protein	Hexosamine content of chondroitin- sulfates	Acid, m.-eq. per gm. protein			
		From hexosamine content		From acid dyes (12)	From basic amino acid con- tent (arginine, histidine, lysine)
		For a mono- basic acid	For a dibasic acid		
	<i>per cent</i>				
Globin.....	10.82	0.843	1.686		1.31 (14) 1.396 (15)
Edestin.....	10.00	0.756	1.512	1.57	1.413 (13)
Gelatin.....	8.00	0.564	1.128	1.03-1.07	1.066 (13)
Egg albumin....	4.78	0.305	0.610		0.649 (13)

The calculations are based on a molecular weight of chondroitinsulfuric acid of (460.3)_n.

the carboxyl group of the uronic acid take part in the salt formation. This is corroborated by the low ash content of the complexes, since 8 per cent hexosamine would correspond to 6.38 per cent Na₂SO₄ if the protein cation had not replaced the Na⁺ of the chondroitinsulfuric acid. (3) The amount of chondroitinsulfuric acid bound by the protein corresponds to the amount of basic amino acids in the protein.

⁵ The complex formed by globin and chondroitinsulfuric acid in neutral solution showed a much decreased hexosamine content, as could be expected from the decreased dissociation of the basic groups at a higher pH.

SUMMARY

The precipitates formed by chondroitinsulfuric acid with some proteins have a constant composition over a fairly wide range of concentration of both components. The acid equivalents of the proteins calculated from the analyses of the compounds agree well in the case of gelatin and edestin with those calculated from salts with acid dyes. They further agree with the basic amino acid content of the proteins studied. They are thus characterized as true salts, in which both the free acid group of the sulfuric acid and the carboxyl of the glucuronic acid take part in the salt formation.

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ON GLYCOPROTEINS

VI. THE PREPARATION OF CHONDROITINSULFURIC ACID

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In the preceding paper, the salt formation of chondroitin-sulfuric acid and various proteins was described. The chondroitinsulfates are stable compounds, soluble in strong alkali or acid, but insoluble in neutral solution.

It was observed that the chondroitinsulfuric acid salt of gelatin was soluble in a concentrated solution of calcium, barium, or strontium chloride. This observation was utilized in the extraction of chondroitinsulfuric acid from cartilage in neutral solution. Hitherto extraction with strong alkali has been employed for the preparation of chondroitinsulfuric acid (1). Jorpes (2) found that 2 per cent NaOH at 0° was the minimum amount necessary to extract the acid from cartilage. Since treatment with alkali might easily lead to decomposition, the present method of extraction by a neutral solution of CaCl₂ seems advantageous.

EXPERIMENTAL

30 gm. lots of the dried cartilage,¹ defatted with acetone and ether and ground to a 40 mesh powder, were mechanically shaken with 300 cc. of 10 per cent CaCl₂ and the extraction was repeated once. The combined turbid solutions were mechanically shaken three times with a chloroform-amyl alcohol mixture (80 cc. of chloroform and 32 cc. of amyl alcohol); the chloroform-protein jelly was separated by centrifuging. The clear supernatant solution was precipitated with twice its volume of alcohol to which a few cc. of glacial acetic acid had been added. The

¹ We thank Dr. David Klein of The Wilson Laboratories, Chicago, for this material.

TABLE I
Composition of Acid Calcium Salt of Chondroitinsulfuric Acid

Analysis	In per cent			In equivalents per equivalent weight			Calculated for acid calcium salt of chondroitinsulfuric acid	
	Preparation I		Preparation III	Preparation II		Preparation III	+2H ₂ O	+3H ₂ O
							per cent	per cent
Nitrogen.....	2.93	2.91	2.98	1.12	1.13	1.25	2.67	2.58
Hexosamine.....	30.4	27.9	28.6	0.89	0.85	0.95	34.2	33.1
Hexuronic acid.....	36.7	36.3	32.2	1.02	1.02	0.99	37.1	35.8
Acetyl.....	8.06	8.03	7.73	1.01	1.02	1.07	8.21	7.94
Sulfur*.....	5.7	6.1	5.6	0.96	1.04	1.05	6.12	5.92
Equivalent weight.....	538	545	594				524	542
Ash.....	9.00	12.64	7.58				13.01 (as CaSO ₄)	12.55 (as CaSO ₄)
Moisture.....	5.8	5.75	10.05					
	degrees	degrees	degrees					
[α] _D of acid calcium salt.....	-17.4	-17.6	-15.9					
[α] _D in neutral solution.....	-31.6	-29.9	-26.6					

* These analyses were made by Mr. William Saschek, of the Department of Biological Chemistry.

precipitate was allowed to stand in the ice box overnight, centrifuged, washed with alcohol, and dissolved in 400 cc. of water. The solution was brought to 0.2 N HCl, 50 gm. of Lloyd's reagent were added, and the mixture was shaken for a few minutes. After centrifugation the residue was washed once with 100 cc. of water and the supernatant solutions were again precipitated by 2 volumes of alcohol. After the precipitate had been washed with alcohol, it was extracted with 100 cc. of water and the clear viscous solution poured into 1000 cc. of glacial acetic acid. A white fibrous precipitate was obtained which, after centrifugation, was washed with alcohol, acetone, and ether and dried. Over 5 gm. of a fluffy powder were obtained; this is the acid calcium salt of chondroitinsulfuric acid. The free acid is not precipitated by alcohol, acetone, or glacial acetic acid, and is unstable in aqueous solution. Table I gives the analytical data² of three such preparations of chondroitinsulfuric acid. It can be seen that the nitrogen is apparently in excess. The hexuronic acid, acetyl, and sulfur values check among themselves and with the equivalent weight. The hexosamine value, however, is too low by 10 to 15 per cent. Since the possibility of the substance being a mixture of polysaccharides is remote, this discrepancy may be attributed to inability to hydrolyze completely the chondroitinsulfuric acid before estimating the hexosamine content. In a previous paper (4) it was shown that the recovery of chondrosamine added to different substances was quantitative after 8 hours hydrolysis. However we found with our preparations of chondroitinsulfuric acid that a longer time (12 hours) of hydrolysis gave better results. The resistance to hydrolysis of chondroitinsulfuric acid was also stressed by Jorpes and Bergström (5).

The preparations all gave a negative biuret reaction in high concentrations. The nitrogen content was not lowered by precipitation with picrolonic acid nor with tannic acid. Tannic acid, which had been used by Mörner (1), did not even cause a turbidity in solutions of our preparation.

The amino sugar of Preparation II was isolated as the hydro-

² The analytical procedures followed are described in Paper III of this series (3). The hexosamine was determined after 12 hours of hydrolysis. All values are corrected for the moisture content determined by drying a 20 mg. sample at 90° to constant weight.

chloride by hydrolyzing 1 gm. for 4½ hours with 20 cc. of 18 per cent HCl containing 0.5 gm. each of SnCl₂ and of BaCO₃. After removal of Sn, BaSO₄, and CaSO₄, 0.2611 gm. (82.5 per cent of the colorimetrically determined amount) of typical chondrosamine hydrochloride crystals was obtained, N (Dumas),¹ 6.27 per cent; $[\alpha]_D$ (at equilibrium) +95.1°.

The similarity in action of CaCl₂ on both the cartilage and the gelatin salt of chondroitinsulfuric acid suggests that the acid is present in the cartilage as a protein salt. This is also brought out by the analysis of the ground native cartilage. Analysis, corrected for 2.63 per cent ash and 7.68 per cent moisture: N, 13.1 per cent; hexosamine, 8.27 per cent; hexuronic acid, 9.51 per cent; acetyl, 3.08 per cent; hexosamine N to total N, 0.046; hexuronic acid to hexosamine, 1.06; acetyl to hexosamine, 1.545. A ratio of hexosamine N to total N of 0.046 compares very well with the corresponding ratio of 0.050 for the gelatin salt of chondroitinsulfuric acid (6).

SUMMARY

A method has been described for extracting chondroitinsulfuric acid from cartilage without the use of alkali. The method involves extraction of the ground cartilage with CaCl₂ solution and removal of the nitrogenous impurities by denaturing with chloroform and amyl alcohol and adsorbing on Lloyd's reagent.

Analytical data are given for the acid calcium salt of chondroitinsulfuric acid thus prepared.

The major portion of the cartilage is a protein salt of chondroitinsulfuric acid.

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¹ This analysis was made by Mr. William Saschek, of the Department of Biological Chemistry.

A STUDY OF "ASCORBIC ACID OXIDASE" IN RELATION TO COPPER*

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The tissue functions of vitamin C from a chemical point of view are still essentially unknown, although many empirical physiological and histological relationships have been clearly established (1, 2). Its sensitivity to aerobic oxidation when copper is present as a catalyst (3, 4) is perhaps its most striking characteristic *in vitro*.

Subsequent to the description of a "hexoxidase" in cabbage leaves by Szent-Györgyi (5), several investigators have reported the existence of an "ascorbic acid oxidase" in other plants and fruits. Such an "enzyme" was reported to be present in apple juice by Zilva (6), in the pods of the drumstick-tree by Srinivasan (7), and in cauliflower juice by Hopkins and Morgan (8). Kertesz, Dearborn, and Mack (9) have studied the destruction by heat of an oxidizing agent in a number of vegetable extracts. Tauber, Kleiner, and Mishkind (10) have reported the concentration of a similar factor from Hubbard squash. Although the latter workers suggested that their "enzyme" was of a different character than the original "hexoxidase" of Szent-Györgyi, the evidence was not conclusive, as shown by the statement of Hopkins and Morgan (8) that there were "insufficient grounds" for distinguishing these enzymes.

Barron, Barron, and Klemperer (11) have questioned the enzymic nature of the catalyst. The more consistent inhibition produced by cyanide compared to 8-hydroxyquinoline on a series

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of vegetable and fruit juices led them to suggest that, "It is . . . quite probable that hemochromogens are the main catalysts for the oxidation of ascorbic acid in these biological fluids possessing no inhibitory mechanisms." McFarlane (12) reported that sodium diethyldithiocarbamate served as a copper inhibitor against the aerobic oxidation of vitamin C in aqueous solution.

An observation in this laboratory that diethyldithiocarbamate completely inhibited the aerobic oxidation of ascorbic acid in cucumber juice, which is very active catalytically, led to a study based on the hypothesis that copper was chiefly responsible for the catalytic effect of "oxidase" preparations.¹ The problem was approached from two points of view: (a) the effect of a series of copper inhibitors upon typical "enzymes," and (b) a comparison of the reported "enzymes" with copper plus proteins.

EXPERIMENTAL

The rate of oxidation of ascorbic acid was measured manometrically in air-filled Warburg vessels. The "enzymes" and copper were invariably placed in the main vessel and ascorbic acid solution in the side arm, with a total volume of 3.3 ml. including 0.3 ml. of 20 per cent KOH in the alkali cup. Unless otherwise indicated, 0.01 mm of ascorbic acid was used, equivalent theoretically to 112 c.mm. of oxygen under standard conditions. To avoid contamination by copper, Kahlbaum's phosphates were employed, and triple distilled water from all-glass Pyrex stills was used for making up solutions and rinsing apparatus. The crystalline ascorbic acid was oxidized very slowly in this kind of water (approximately 5 c.mm. of O₂ per hour). Experiments were performed at 37°. The rate of reaction was expressed as c.mm. of O₂ per hour, extrapolated when necessary from the linear rate obtained for the major part of the reaction.

The rate of catalysis established by copper-abumin or copper-gelatin mixtures was approximately proportional to the concentration of copper and inversely proportional to the concentration of protein (*cf.* Fig. 1).

The experimental protein solutions contained sufficient copper

¹ The authors wish to thank Dr. M. O. Schultze for the copper analyses reported in this paper, and Professor E. B. Hart for the samples of copper amide biuret and copper hematoporphyrin.

to give an oxidation rate comparable to that of the "enzymes." As shown in Table I, the concentration of copper in the enzyme preparation was about the same as that in the copper-gelatin solutions. In the albumin solutions the copper concentration was higher. The method of Fischer and Leopoldi (13) was used in making copper analyses.

Although there was sufficient copper in some of the buffers to cause a slow oxidation of ascorbic acid, this effect was negligible in the presence of protein. No difference in the rate of oxidation

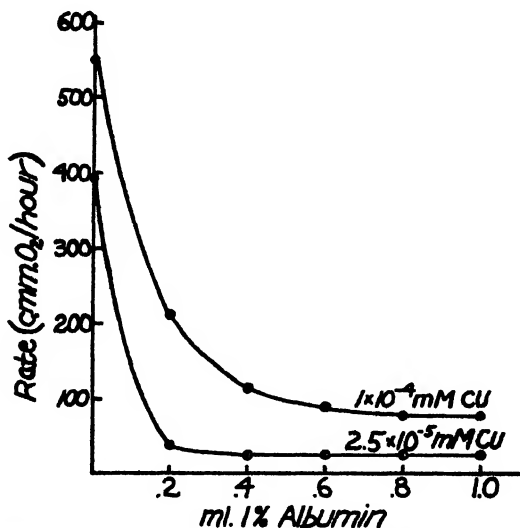


FIG. 1. Effect of ovalbumin on copper catalysis of the oxidation of ascorbic acid. Ascorbic acid, 0.01 mM, pH = 5.8, $M/15$ PO_4 buffer, $T = 37^\circ$.

by the "enzymes" or copper-albumin could be detected when small or large quantities of buffer were used.

Preparation of Enzymes—Although only a few experiments dealt with cabbage and cucumber press-juice, "purified squash oxidase" and cauliflower press-juice were studied in more detail.

The final dried acetone precipitate of squash "oxidase," prepared as described by Tauber, Kleiner, and Mishkind (10), was only slightly soluble in water. Although the soluble portion was active, boiling for 5 minutes did not destroy the activity. Furthermore, the greater part of the activity was associated with material that was coarsely suspended as altered protein. For this reason

it was preferred to suspend the final acetone precipitate in water, aerate until free of acetone, and utilize this preparation as squash "oxidase." By determining the units of activity in the alcohol extract and the final product it was found that the preparations were of equal strength to those of the original investigators.

Effect of Inhibitors—Seven inhibitors that have been used for qualitative and quantitative tests for copper were examined for their inhibitory power on the press-juice of cabbage and cauliflower, the squash "oxidase" described above, copper, copper-albumin, copper-gelatin, and nicotine-hemochromogen. The inhibitors were neutralized if necessary and diluted to the desired concentrations with $M/15$ Sørensen buffer (pH 6.0). Table I records the rates of oxygen consumption produced by the unpoisoned catalysts, while the rates in the presence of poisons are more conveniently recorded as per cent inhibitions.

The inhibitors, all of which poisoned the catalytic action of copper, copper-albumin, and copper-gelatin, had a similar effect upon the catalytic activity of cauliflower juice and purified squash "oxidase." It may be noted that the relative effect on cabbage juice was considerably less than on squash "oxidase," and somewhat less on cauliflower juice (particularly 8-hydroxyquinoline). This finding is probably due in greater degree to the higher protein content of the preparations than to the presence of non-copper catalysts. That protein is able to lower the effective concentration of inhibitor was demonstrated in an experiment with high and low concentrations of albumin. With 1 ml. of copper + 1 ml. of 1 per cent albumin as the catalyst, diethyldithiocarbamate and 8-hydroxyquinoline produced inhibitions of 92 and 94 per cent respectively, but with 1 ml. of copper + 1 ml. of 6 per cent albumin as catalyst, there was only 40 and 45 per cent inhibition. The latter protein concentration corresponded approximately to the per cent of solids present in the cauliflower press-juice, whereas squash "oxidase" contained considerably less solids. Therefore the amount of inhibitor used was increased to the quantities reported in Table I, although in nearly all cases much less inhibitor produced comparable effects. The effect of these higher concentrations of inhibitors could hardly be due to a non-specific blocking of an enzyme surface, since (a) the seven compounds selected had copper-combining capacity as their only common

characteristic, and (b) much higher concentrations of non-specific inhibitors such as urethane and sodium fluoride had little or no effect.

TABLE I
Effect of Copper Inhibitors

Ascorbic acid, 0.01 mM; $T = 37^\circ$; pH = 6.0 ± 0.1 .

Inhibitor	Catalysts						
	Cu, 3×10^{-4} mM	Cu- albumin, 2×10^{-4} mM Cu	Cu- gelatin, 6×10^{-4} mM Cu	Nicotine- hemo- chromo- gen, 3×10^{-4} mM hemin	Cauli- flower press- juice, 5×10^{-3} mM Cu	Squash "oxi- dase," 3×10^{-3} mM Cu	Cab- bage juice
	Rate of O_2 consumption, c.mm. O_2 per hr.						
None	420	260	240	320	220	290	90
	Per cent inhibition						
Diethyldithiocarba- mate, 3×10^{-3} mM*	100	96	98	0	76	100	68
8-Hydroxyquinoline, 7×10^{-3} mM	99	92	96	0	42	94	62
Pyridine, 1 mM, KCNS, 1 mM	92	90	98	-37†	84	85	50
Sodium cyanide, 0.04 mM	98	95	97	48	88	95	56
Potassium ferrocya- nide, 0.025 mM	93	87	90	0	58	89	32
Potassium ethyl xan- thate, 0.06 mM ...	95	78	88	0	83	97	68
Sodium sulfide, 0.025 mM	98	86	85	8	91	96	35

* The quantities of copper, ascorbic acid, and inhibitors recorded in this table represent the total amounts in the 3 cc. volume of reactants. The total amount of albumin and gelatin in 3 cc. was 10 mg.

† 37 per cent increase in O_2 consumption rate.

The results with inhibitors are somewhat different from the results reported by Barron *et al.* (11) in which cabbage and squash juice were not inhibited by 8-hydroxyquinoline, although markedly affected by cyanide. The apparent discrepancy may be due

to different inhibitor to protein ratios in the preparations used. Cyanide is an active inhibitor against both copper and hemochromogen. The results recorded in Table I show that nicotine-hemochromogen was not affected by the series of inhibitors used. The high concentration of pyridine in pyridine-KCNS apparently produced a more effective concentration of pyridine hemochromogen.

Effect of Heat—Although the catalytic power of copper salts is not inhibited by boiling, the "enzymes" are heat-sensitive. Copper-albumin showed an 84 per cent decrease in its catalytic power after 5 minutes heating at 100°, parallel with a 75 to 85 per cent destruction of the cauliflower, squash, and cucumber "enzymes." That this phenomenon is coincident with coagulation of protein is suggested by (a) the heat stability of the non-coagulable copper-gelatin, (b) the stability of cucumber "enzyme" at 55° (no coagulation) and its destruction at 100° (coagulation), and (c) the fact that 88 per cent of the original copper was found by analysis in the heat-precipitated cauliflower protein, and 48 per cent in the case of squash. Since this precipitated copper is practically inactive, the heat coagulation of protein must involve a binding of the copper in a non-catalytic form. It is also of interest that albumin, when heat-coagulated in the absence of copper, has a greater inhibitory effect on copper catalysis than the original protein. We have not studied the possible relationship of the above phenomena to sulfhydryl groups.

Optimum pH—In their original paper Barron, DeMeio, and Klemperer (4) showed that the catalytic power of inorganic copper was greatly dependent upon the pH, but that in the non-oxidizable range of ascorbic acid (below pH 7.6) there was no distinct optimum. The use of citrate-phosphate buffer in the presence of copper did not alter the above relationship.

This relation is greatly changed, however, in the presence of albumin or gelatin, in which case a distinct optimum pH range was found, comparable to that of the "enzymes." Fig. 2 illustrates the similarity between the postulated enzymes and the copper-protein complexes.

Kinetics—Szent-Györgyi (5) found, with cabbage "oxidase," an increase of velocity with increasing substrate concentration, and postulated a complex reaction involving an intermediate sub-

stance X. Since Tauber *et al.* (10) found, with their squash "oxidase," that no increase in velocity occurred with increasing substrate concentration, they concluded that their "enzyme" was of different character than that of Szent-Györgyi. Since both findings are in harmony with the Michaelis-Menten theory, their difference may depend only on the substrate to "enzyme" ratio.

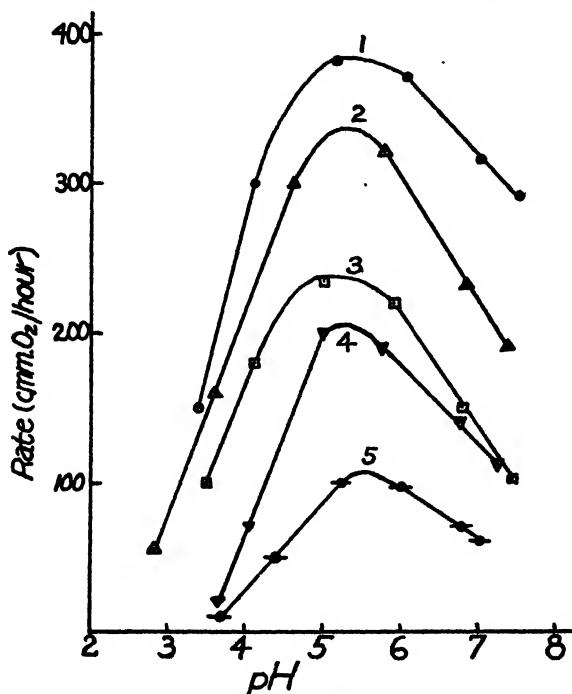


FIG. 2. Optimum pH of preparations. Ascorbic acid, 0.01 mm, $T = 37^\circ$, PO_4 -citrate buffer. Cu in Cu-albumin, 3×10^{-4} mm (total); in Cu-gelatin, 6×10^{-4} mm. Albumin and gelatin, 10 mg. total. Curve 1, copper-albumin; Curve 2, squash "oxidase;" Curve 3, copper-gelatin; Curve 4, cauliflower juice; Curve 5, cabbage juice.

The results given in Fig. 3 show that it is possible to duplicate Szent-Györgyi's results with squash "oxidase." The effect of changing the substrate to copper-albumin ratio is also shown in Fig. 3. It will be noted that both types of curve may be obtained in accordance with the variation in this ratio.

It has been noted before that the rate of oxidation of ascorbic acid follows a linear course until the vitamin has almost completely disappeared. Hopkins and Morgan (8) have suggested that, "it is perhaps necessary to assume that it [the enzyme] activates both the reduced and oxidized molecules of ascorbic acid." The same phenomenon is noted, however, in the presence of copper-protein. Such velocity relations are complex in the higher pH ranges (6.0 to 7.6) because the irreversible oxidation becomes

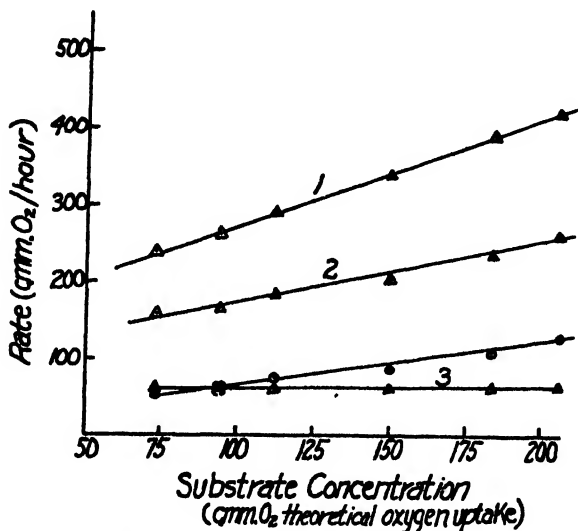


FIG. 3. Velocity-substrate relation. pH = 6.0, $T = 37^\circ$. Δ copper-albumin, \odot squash "oxidase." Albumin, 10 mg. total. Curve 1, 4×10^{-4} mM of Cu total; Curve 2, 1×10^{-4} mM of Cu total; Curve 3, 1×10^{-5} mM of Cu total.

increasingly prominent. In fact, at pH 7.4 the oxygen consumption of a copper-catalyzed vitamin solution was nearly twice that indicated by simultaneous indophenol titration of the same solution. This continued and irreversible oxidation superimposed on the reversible oxidation (equivalent to $-2H$) makes the velocity of the latter difficult to measure and interpret.

Inactivation by Acid—Tauber, Kleiner, and Mishkind (10) found that purified squash "oxidase" was inactivated at pH 2.0.

Squash "oxidase," cauliflower juice, and copper-albumin were

adjusted to pH 2.0 and allowed to stand for 2 hours. They were then tested for their catalytic activity at pH 6.0 and compared with the untreated preparations at the same dilution. The inactivations amounted to 75, 68, and 64 per cent for squash, cauliflower, and copper-albumin respectively. Cauliflower juice was partially inactivated at pH 3.0. Acid inactivation of the preparations appears comparable to heat coagulation, a process in which copper is bound in a non-catalytic form by the denatured proteins.

Tryptic Digestion—Tauber *et al.* (10) also observed a progressive decrease in the activity of their preparation due to the action of trypsin.

Triplicate 90 hour digestions at 37° were carried out on squash "oxidase," cauliflower juice, and copper-albumin with 0.5 per cent trypsin (Fairchild Brothers and Foster) at pH 6.8 to 7.2. Samples of the tryptic digests were tested for their catalytic power at four different time intervals. The results were not so consistent as those indicated by the above workers. The maximum inhibitions obtained were 40 per cent in the case of squash, 18 per cent with cauliflower, and practically none with copper-albumin. If copper were liberated from the protein by digestion of the latter, one might expect either no decrease in catalytic activity or an increase. A significant result was obtained, however, upon testing the effect of small amounts of the tryptic digests on the catalytic power of copper. The results recorded in Table II indicate that the products of proteolysis of the squash and cauliflower proteins inhibit copper catalysis to a greater extent than the original material. Digested albumin showed little change in its inhibitory effect on copper.

Although copper-albumin showed no decrease in activity upon digestion, neither did its products of proteolysis inhibit added copper more than the original material. The latter observation has also been made with albumin containing no copper during digestion.

Combined Forms of Copper—Evidently the linkage between copper and protein is one in which copper is free to undergo alternate oxidation and reduction and to combine with copper inhibitors. In this connection the findings of Ettisch, Sachse, and Beck (14) and of Borsook and Thimann (15) are of interest in

demonstrating the formation of un-ionized complexes with proteins and amino acids. The following points in our work are of interest in relation to the catalytic action of combined copper: (a) Cauliflower press-juice assumed a brown color when diethyldithiocarbamate was added, indicating the formation of a complex similar to that formed with inorganic copper salts. The juice lost practically none of its activity, however, when dialyzed for as long as 24 hours at pH 4.0 to 7.5. (b) Albumin and gelatin in equal weights possessed considerably different inhibitory powers on the copper catalysis of ascorbic acid oxidation. The lesser inhibitory power of gelatin is of interest in connection with its low sulfur content and non-coagulability. (c) Copper amide biuret (16, 17), a combined form of copper, was found to catalyze

TABLE II

Inhibition of Copper Catalysis by Original and Digested Preparations

Ascorbic acid, 0.01 mM; $T = 37^\circ$; pH = 6.1.

Digested material	Rate of O_2 consumption, c.mm. O_2 per hr.			
	Original	Digested	Original + Cu (1×10^{-4} mM)	Digested + Cu (1×10^{-4} mM)
Squash "oxidase"	88	55	142	63
Cauliflower juice	61	51	102	66
Copper-albumin	44	48	88	80

the oxidation of ascorbic acid at the same rate as an equimolar amount of inorganic copper in the pH range of 4.0 to 7.5. It was inhibited quantitatively by diethyldithiocarbamate (2 moles of inhibitor per mole of Cu), with the formation of a typical brown copper complex. (d) Copper hematoporphyrin (16), unlike hemin, was able to function as a catalyst for the oxidation of ascorbic acid. The catalytic action of this "bound" copper was also completely inhibited by diethyldithiocarbamate. (e) The usual activity of glutathione in blocking the catalytic activity of copper is decreased rapidly as the concentration of protein increases.

DISCUSSION

It appears from our investigations that copper may be combined with proteins in such a manner that it retains its catalytic rôle in

the oxidation of ascorbic acid. It is not assumed that the copper-albumin mixture that we have studied is the exact counterpart of the copper-protein present in vegetable preparations, but rather, that the properties of such mixtures and complexes provide an experimental basis for understanding the catalytic behavior of minute quantities of copper in natural products.

Although more copper was required to produce a given catalytic effect in the presence of ovalbumin than was found in the vegetable press-juices, copper + gelatin and copper + edestin proved to be nearly like the vegetable juices in this respect. Furthermore, a large fraction of the copper in the copper-albumin mixture was dialyzable, whereas that in the "enzymes" was not. On the other hand, the proteins present in the "enzyme" preparations possessed an efficiency comparable with albumin in the manner in which they protected ascorbic acid from oxidation by added copper.

The concept of a specific "oxidase" for the oxidation of ascorbic acid has been a factor in the belief that this vitamin is an important catalyst in biological oxidation. The view that copper may serve as this "enzyme" does not, *per se*, argue for or against such a rôle for the vitamin.

The evidence given concerning the copper nature of the catalyst in vegetable juices has an immediate significance in relation to the preservation of the vitamin in foods and in vitamin analysis, but further study will be necessary to find whether there is a relationship between the vitamin and copper in living tissues.

SUMMARY

1. The catalytic activity of squash and cauliflower juices on the oxidation of ascorbic acid, previously ascribed to a specific "oxidase," is attributed to the copper present in combination with protein material.

2. Seven copper inhibitors produced nearly complete poisoning of the "enzymes," as well as of inorganic copper and copper-protein mixtures. These inhibitors did not affect the catalytic function of nicotine-hemochromogen in a manner which would suggest that this type of substance is responsible for the oxidation of ascorbic acid by cauliflower juice or "purified squash oxidase."

3. The catalytic properties of copper are changed greatly by the

presence of proteins. A mixture of copper and albumin assumes the characteristic properties of the "enzymes." It displays an optimum pH similar to that of the "enzymes," is inactivated by heat and acid, and shows similar velocity relations to the quantity of substrate.

4. The type of union which may exist between copper and protein has been discussed and the catalytic ability of "bound" copper illustrated by copper amide biuret and copper hematophorphyrin.

5. Although the above conclusions apply only to cauliflower juice, "purified squash oxidase," cucumber juice, and cabbage juice, it is suggested that the other "ascorbic acid oxidases" described in the literature are probably not essentially different from these. A study of several other vegetable and fruit juices is in progress in this laboratory.

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THE METABOLISM OF THE ORGANIC ACIDS OF THE TOBACCO LEAF DURING CULTURE*

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Although ten or more different organic acids have been detected in the leaf of the tobacco plant (for literature see (1)), by far the greater part of the organic acidity of this tissue is due to the presence of malic, oxalic, and citric acids. The relative proportions of these three substances vary between fairly wide limits, but, in general, approximately 20 per cent of the organic solids and 80 per cent of the organic acidity of the so called Connecticut shade-grown tobacco leaves we have chiefly studied can be ascribed to them. The total quantity of acids is of a magnitude comparable with the total quantity of protein present in the tissues, and the transformations that they may undergo therefore rank, from the quantitative point of view at least, among the most significant for our understanding of the general metabolism of leaf tissues.

The whole subject of the organic acid metabolism of green plants is in a most unsatisfactory condition not only from the factual, but also from the theoretical standpoint. The literature of the subject deals chiefly with fluctuations in the titratable acidity rather than with determinations of specific acids, although conclusions regarding the metabolic changes of individual substances have been drawn. The chief evidence adduced in support of such conclusions has been merely that the individual acid in question is the predominating acid of the tissue under investigation. The theoretical interpretations of the data that have been advanced are almost entirely speculative, and it is probable that the metabolic relationships have in few cases been definitely

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

established. For the most part organic acids appear to be more closely related to the carbohydrate metabolism than to the protein metabolism, but even this broad generalization can be specifically applied only to lower organisms.

The entire problem has recently been carefully reviewed by Bennet-Clark (2) and reference should be made to his papers for a comprehensive statement of the present position of our knowledge of the organic acids in plant physiology. This is a field in which investigation is still largely in the fact-finding stage of development, and advances in the immediate future are to be looked for chiefly in the direction of the application of new and more specific methods of analysis.

It is with this in mind that much time has been devoted in this laboratory to a consideration of the behavior of the organic acids during culture of leaves in water or nutrient solutions in light and in the dark. A series of samples of excised tobacco leaves of approximately equal fresh weight were cultured in distilled water, in dilute glucose solution, or in a nutrient salt solution that contained ammonium sulfate as the source of nitrogen, duplicate sets being exposed to continuous light or to continuous darkness. Control samples analyzed at the start, and samples removed for analysis from time to time permitted the changes in composition of the tissues to be followed. The general technique has been fully described in a previous publication (3), and some of the data that concern the nitrogenous constituents of these samples are given in another paper (4).¹

The methods of analysis employed have been described in recent papers from this laboratory (6); the results have been calculated in milli-equivalents of acid per kilo of fresh weight of the leaf tissue before being subjected to culture. On the assumption that the individual samples had the same composition at the start of the operation, it is clear that changes in quantity of any constituent later found must represent chemical changes that occurred during the culture period. The validity of this conclusion rests upon the degree with which the different samples actually duplicated each other. Every effort was made to select samples of leaves as closely alike as possible, and study of constituents which

¹ A more detailed description of these experiments than is here possible is to be given in Bulletin 399 (5) from this Station.

should not be expected to change during the culture period indicates that the variations due to sampling error were not excessive. Thus the total nitrogen of one series of samples (1935, LW) gave a mean value of 3.96 gm. per kilo of original fresh weight with a probable error of ± 0.04 , the highest value being 4.16 gm. and the lowest 3.66 gm. It would therefore seem that changes of any constituent that exceed ± 10 per cent of the quantity originally present have real significance.

The leaves subjected to this procedure were obtained from field crops grown during the past 3 years. The designations of the sets of samples refer to the conditions of the culture; the letters D and L indicate culture in the dark or in light, and the letters W, G, and N refer to culture respectively in water, glucose, or nutrient salt solution containing ammonium sulfate. Where necessary, the date has been prefixed to the sample designation.

Results

The data for total organic acidity are obtained by electro-metric titration between the arbitrarily selected limits pH 7.8 to 2.6 of a solution prepared by exhaustive ether extraction of the dried and acidified tissue. Corrections are applied to allow for the facts that oxalic acid reacts with only one carboxyl group, and malic and citric acids with only approximately 90 per cent of their acidity, and it is assumed that the unknown acids included in the titration value behave similarly to these two. Table I gives a part of the data from six culture experiments on leaves collected in 1935 and shows that the changes in the total organic acidity, either in light or in darkness, are of a minor nature. In one experiment in the light there was a probable increase, and in two in the dark a probable slight decrease, but the relative order of magnitude of the change is small in all cases.

The oxalic acid content of the tissues, regardless of conditions, was likewise constant within the limits of accuracy of the method throughout the period of culture, as is shown in Table I. This result is perhaps to be expected, as the oxalic acid of tobacco leaves is present largely in a hot water-insoluble form, probably as the insoluble calcium salt (7). The inference cannot be drawn, however, that oxalic acid is in general an inactive end-product of metabolism. As we have previously shown, oxalic acid is in

part withdrawn from the leaves along with other acids during the period of maturation of the seed (8).

The behavior of the malic acid during culture of the leaves in light calls for little comment; the data of the LG experiment are not satisfactory inasmuch as they show a marked drop succeeded by a rise above the original level, the final value being a little less than that at the start. Whether these changes are real, or are the effect of sampling errors, cannot be decided. The net change over the entire period is, however, small, and the data

TABLE I

Total Organic Acidity, Oxalic, Malic, and Citric Acids, of Tobacco Leaves

The figures are given in milli-equivalents per kilo of original fresh tissue.

Cul- ture	Total organic acidity					Oxalic acid				
	0 hr.	78 hrs.	143 hrs.	235 hrs.	Δ	0 hr.	78 hrs.	143 hrs.	235 hrs.	Δ
LW	311	357	372	367	+56	36.8	36.8	38.3	39.1	+2.3
DW	311	308	281		-30	36.8	41.0	38.2		+1.4
LG	326	326	346	316	-10	40.6	41.1	41.0	38.7	-1.9
DG	326	288	271		-55	40.6	40.7	40.1		-0.5
LN	248	233	241	247	-1	36.9	36.1	39.2	39.3	+2.4
DN	248	233	243		-5	36.9	37.0	41.4		+4.5
	Malic acid					Citric acid				
	0 hr.	78 hrs.	143 hrs.	235 hrs.	Δ	0 hr.	78 hrs.	143 hrs.	235 hrs.	Δ
LW	226	242	245	243	+17	50.8	62.7	54.7	63.1	+12.3
DW	226	98	67		-159	50.8	111	148		+97.2
LG	232	188	252	193	-39	15.9	54.6	30.6	45.0	+20.1
DG	232	141	84		-148	15.9	73.4	107		+91.1
LN	151	135	145	136	-15	34.9	25.2	18.5	18.7	-16.2
DN	151	82	54		-97	34.9	54.7	96		+61.1

of the other two experiments in light make it clear that the change in malic acid content is a minor matter. Culture of tobacco leaves in the dark, however, brings about a profound decrease in the quantity of malic acid present, the percentage loss being 70, 64, and 64 per cent of that originally present during a period of 143 hours. This represents the disappearance of 10.6, 9.9, and 6.5 gm. of malic acid respectively in the three experiments, or 14.2, 11.2, and 8.5 per cent respectively of the original organic solids of the leaves. Thus the disappearance of malic

acid during culture in the dark is quantitatively one of the most important reactions that occurs.

The changes in the citric acid are also given in Table I. The three cultures in light again show changes of minor importance, although the data of the LG experiment are not entirely satisfactory: the initial value is unusually low, and the fluctuations are similar to those of the malic acid, though in the reversed sense. But the rapid formation of citric acid during culture in the dark is striking; in the three experiments 6.2, 5.8, and 3.9 gm. respectively of this substance are synthesized, these amounts being the equivalent of 8.3, 6.6, and 5.1 per cent of the organic solids of the tissues. This change therefore also involves a very material proportion of the organic substances present.

TABLE II

Malic Acid and Citric Acid of Tobacco Leaves Cultured in Water in Light, in Dark, and with 24 and 48 Hours Alternations of Light and Dark

The figures are given in milli-equivalents per kilo of original fresh tissue.

Culture	Malic acid			Citric acid		
	0 hr.	120 hrs.	240 hrs.	0 hr.	120 hrs.	240 hrs.
Continuous light (LW).....	185	191	180	26.7	36.0	50.3
" dark (DW)....	185	62	(30)*	26.7	144	(140)*
24 hr. alternation.....	185	125	109	26.7	64.5	74.6
48 " "	185	137	105	26.7	58.6	91.0

* Not regarded as valid controls after 120 hours of culture.

The data for the unknown organic acids are obtained by subtraction of the sum of the three determined acids from the total organic acidity; the figures are accordingly affected by a considerable experimental error and need not be given in detail. Both the LG and DG experiments are unsatisfactory, but the other four suggest an increase of acidity of the order of magnitude of 25 milli-equivalents. This quantity may or may not have significance.

The phenomenon of a marked decrease in malic acid with a corresponding increase in citric acid during culture of tobacco leaves in the dark has been verified by experiments carried out with leaves collected in 1934 and again in 1936. The more recent experiments involved study not only of leaves cultured in water continuously in the dark and in light, but also of leaves that were

cultured both in dark and in light for alternating periods of 24 and 48 hours duration. As might be anticipated, this alternation brought about changes in the acids intermediate between the two extremes. The data are shown in Table II. The leaves employed were somewhat younger and smaller than those used in the previous experiments, and were probably at a more vigorous stage of growth, which may account for the definite increase in citric acid that occurred in light.

The leaves cultured continuously in the dark were still in good condition at the end of 120 hours, but subsequently lost water rapidly and became completely yellow; the other sets of samples remained in fairly good condition throughout. It will be noted that the citric acid in the dark control set reached its maximum value in 120 hours, and that this substance was not materially affected by the subsequent autolytic changes. Malic acid, however, continued to decrease throughout the period of culture. The dark control samples can be regarded as valid controls only during the first 120 hours of culture, and for that reason the data at 240 hours in this experiment are enclosed in parentheses.

The magnitude of the changes in malic and citric acids during 120 hours of culture in the dark is striking. The leaves contained initially 12.4 gm. of malic acid per kilo of fresh weight, or 20.0 per cent of the organic solids. During culture, they lost 8.2 gm. of malic acid or 13.3 per cent of the organic solids, and gained 7.5 gm. of citric acid or 12.1 per cent of the organic solids.

The data on the samples exposed alternately to light and dark indicate that the changes were intermediate in magnitude between the controls, and the detailed data, when plotted, give curves which show alternations in slope in the sense that would be expected. The intervals between analyses were unfortunately too long to reveal the alternations in minute detail, and consequently only the data that show the main features of the changes have been included.

The experimental results that have been obtained on tobacco leaves can be reduced to the statement that the three chief organic acids undergo only minor changes in amount during culture in light; in the dark, however, in spite of the fact that the total organic acidity remains substantially unchanged, there is a profound loss of malic acid and an equally striking increase in

citric acid. The observed behavior thus strongly suggests that a conversion of malic to citric acid took place under these circumstances.

DISCUSSION

Before we proceed to the discussion of this assumption, it is necessary to consider another possible fate of malic acid. The literature contains many suggestions that asparagine arises from malic acid with fumaric acid as an intermediate (9), the evidence being chiefly that these substances are frequently found together in the same tissues and are related in chemical structure. The present experiment, however, furnishes evidence that is clearly against this view. It has been shown elsewhere (4) that a con-

TABLE III

Relation between Malic Acid Loss and Asparagine Gain

The figures not otherwise designated are given in gm. per kilo of original fresh tissue.

Samples	Time elapsed	Asparagine amide N increase	Asparagine increase	Malic acid equivalent	Actual malic acid loss
	hrs.	gm.	gm.	gm.	gm.
1935, DW.....	143	0.302	2.85	2.89	9.65
1935, DG.....	143	0.285	2.69	2.73	10.1
1935, DN.....	143	0.383	3.61	3.67	6.5
1936, DW .. .	120	0.218	2.06	1.99	8.2

siderable synthesis of asparagine occurred during culture in the dark. Table III gives the quantities that were formed and, if it is assumed that the carbon compound from which the asparagine is derived is malic acid, the quantities in the next to the last column would have been used. The actual malic acid losses shown in the last column are much greater, and there is no simple relationship between the increase in asparagine and the loss of malic acid in the different experiments. Even if a part of the malic acid were utilized in this way, most of it must have had some other fate. Furthermore, this conversion would imply a diminution in the total acidity, unless some other reaction occurred to compensate for it, and such a diminution was not observed. Finally, there was nearly as much asparagine synthesized

during the more protracted light cultures as was formed in the dark, but no substantial alteration in the malic acid content occurred. Accordingly a direct genetic connection between malic acid and asparagine may be dismissed as highly improbable in the present case.

In order to assess the probability of a conversion of malic to citric acid, it will be convenient to consider possible sources of the citric acid that was formed during the culture of these leaves in the dark. Both proteins and carbohydrates may be regarded as sources as well as the organic acids. During culture, a material part of the leaf protein underwent digestion with the liberation of free amino acids which were extensively deaminized and, accordingly, a considerable quantity of carbon compounds must have become available for subsequent chemical changes. The losses of protein nitrogen in the 1935 DW, DG, and DN experiments were respectively 1.27, 1.15, and 1.16 gm. Multiplication by the conventional factor 6.25 and subtraction of the nitrogen, since we are interested only in non-nitrogenous material, leaves 6.6, 6.0, and 6.1 gm. of organic solids of protein origin possibly available for citric acid synthesis. These roughly estimated quantities are of a sufficiently large order of magnitude, especially if one considers that oxygen must be added in order to effect the synthesis. But in the 1936 experiment, the loss in protein nitrogen in 120 hours was 1.03 gm., which could provide only 5.4 gm. of non-nitrogenous organic solids. This is hardly adequate to account for the synthesis of 7.6 gm. of citric acid even on the most favorable assumptions of the efficiency with which so complex a mixture of organic compounds could be converted into the necessary precursors of citric acid. Entirely aside from the questions raised by the chemical structure of the substances derived from the protein, there is insufficient organic material to give rise to this intrinsically highly improbable conversion. Moreover, very little synthesis of citric acid occurred in the light, in spite of the fact that nearly as large a quantity of protein decomposition products was rendered available.

The possibility of a conversion of soluble carbohydrates to citric acid is even more unlikely, as can be most clearly shown from data obtained in connection with the 1936 experiment. The fresh leaves at the start contained 1.5 gm. of soluble reducing

carbohydrate, calculated as glucose, of which approximately 1.1 gm. were fermentable sugar. During culture for 120 hours in the dark, this reducing carbohydrate disappeared almost completely. The leaves contained only 0.1 gm. of starch at the start, and this also promptly disappeared. There was consequently far too little soluble carbohydrate in these samples to account for the observed increase in citric acid. A similar situation obtained in the samples of the 1935 experiments.

The soluble carbohydrate increases in the leaves cultured in light were, on the other hand, remarkably large. The reducing carbohydrate (1936, LW) increased from 1.5 gm. to 13.7 gm. in 120 hours and to 18.4 gm. in 240 hours. Of these quantities 10.2 and 13.6 gm. respectively were fermentable sugar. The citric acid did, in fact, increase by nearly 100 per cent of the amount originally present (see Table II), but this corresponded to an increase of from only 1.7 to 3.2 gm. per kilo of tissue over the whole period—a minor change compared to the increase in sugar. There is no evidence with respect to its origin. Furthermore, there was no increase in malic acid, which suggests that the reactions that lead to carbohydrate synthesis do not necessarily lead to malic acid synthesis.

One further point should be mentioned. Leaves cultured in the dark undergo a profound loss of organic solids. The leaves of the 1936 DW culture contained 61.7 gm. of organic solids at the start, but only 44.6 gm. after culture for 120 hours. Thus 17.1 gm. or 27.7 per cent of the organic solids underwent metabolic changes which resulted in the formation of volatile products that escaped from the tissues. In spite of this loss, the asparagine increased by 2.06 gm., and the citric acid by 7.6 gm. These definitely established changes alone involved 43 per cent of the entire quantity of organic matter present. It is obvious that there can be little organic material present in the tissues that was not in some way affected.

It has been shown that the synthesis of citric acid is probably not associated with changes in the protein or in the soluble carbohydrates. To argue that the malic acid must therefore have been involved is to imply that no other possibility remains open and this, in the present state of our knowledge of plant composition, is by no means certain. On the other hand, the chemical

facts at our disposal are decidedly in favor of this conclusion. In the first place, the total organic acid acidity was unchanged during the extensive loss of malic acid and formation of citric acid. Within reasonable limits, therefore, the citric acid must have arisen from a compound which is itself an acid. Furthermore, the structural relations of the compounds involved lend considerable support to this view. Knoop and Martius (10) have recently shown that pyruvic acid will condense with oxalacetic acid at room temperature in alkaline solution to form a product

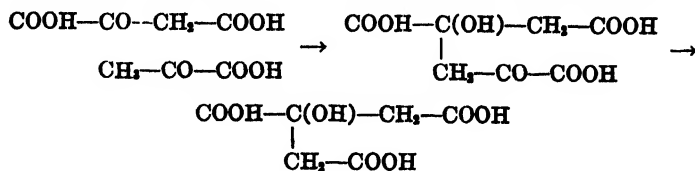
TABLE IV

Relation between Malic Acid Loss and Citric Acid Gain

The figures not otherwise designated are given in gm. per kilo of original fresh tissue.

Samples	Time elapsed hrs.	Malic acid loss	Citric acid gain	Ratio, citric to malic acid	Citric acid gain calculated
		gm.	gm.		gm.
1935, DW.	143	10.66	6.20	0.582	7.63
1935, DG.	143	9.92	5.82	0.586	7.10
1935, DN.	143	6.54	3.91	0.598	4.68
1934, DW.	279	8.82	7.58	0.859	6.31
1936, "	120	8.2	7.5	0.915	5.87

which, on oxidation with hydrogen peroxide, gives rise to citric acid in 35 per cent yield. They formulate the reaction as follows:



If malic acid may be assumed to undergo reduction to oxalacetic acid in the tissues under the action of malic dehydrogenase (11), one of the necessary reactants is provided. Pyruvic acid may arise either by decarboxylation of this substance, or as a by-product of the carbohydrate metabolism. If the former alternative occurs, 2 moles of malic acid should give a yield of 71.6 per cent of citric acid. In the last column of Table IV are shown the quantities of citric acid which would be formed from malic

acid on these assumptions. In the first three experiments, the actual citric acid synthesis was less than the quantity so calculated, in the others it was greater. There is a general agreement in the relative orders of magnitude, however, and when it is remembered that the pyruvic acid required for the conversion may arise from the carbohydrate which disappeared during the culture period, the agreement of the results with the hypothesis is perhaps all that could be expected.

SUMMARY

The three chief organic acids of the tobacco leaf, malic, citric, and oxalic acids, undergo very little change in absolute amount during culture of the leaves in the light, although extensive photosynthesis occurs. The total organic acidity likewise remains essentially constant. During culture in the dark, however, the quantity of malic acid diminishes profoundly and the quantity of citric acid increases. The oxalic acid and the total organic acidity both remain unchanged.

A consideration of the possible sources from which the newly formed citric acid may be derived indicates that malic acid is the most probable. The quantity of soluble carbohydrate present is quite inadequate to account for it, and there is little likelihood on chemical grounds that it may be derived from the protein. A mechanism founded upon the observations of Knoop and Martius, whereby malic acid may be converted into citric acid, is suggested, and it is shown that the quantities of acids involved in the interchange are such that this hypothesis is a reasonable explanation of the results.

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THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

XLVIII. THE OCCURRENCE OF PHTHIOCEROL IN THE WAX FROM VARIOUS STRAINS OF THE HUMAN TUBERCLE BACILLUS*

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A dihydric alcohol named phthiocerol of the formula $C_{28}H_{72}O_2$ or $C_{28}H_{74}O_2$ was isolated recently by Stodola and Anderson (1) from wax fractions of the human tubercle bacillus, Strain H-37. Phthiocerol is a crystalline substance which can be purified and identified quite readily, hence it might serve as a convenient means of differentiating between the human tubercle bacillus and other acid-fast bacteria. Investigations in this laboratory have shown that the waxes isolated from the timothy-grass bacillus (2), the leprosy bacillus (3), and the avian tubercle bacillus (4) do not contain phthiocerol but two other higher alcohols, namely *d*-eicosanol-2 and *d*-octadecanol-2. It remained, however, a question of importance to determine whether phthiocerol is a constant component of the wax from all strains of the human tubercle bacillus.

The results reported by Crowder, Stodola, Pangborn, and Anderson (5) on the lipid fractions of five different strains of the human tubercle bacillus indicate that large differences exist in the lipid content as well as in the chemical constants of the lipids, although the five strains were grown under identical conditions on the Long (6) synthetic medium. We have now examined the wax fractions obtained from four of the recently isolated strains of the human tubercle bacillus, Strains A-10, A-12, A-13, and

* The present report is a part of a cooperative investigation on tuberculosis; it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

† Holder of a National Tuberculosis Association Fellowship at Yale University, 1936-37.

A-14, for the presence of phthiocerol. The results of this examination are clear and definite; phthiocerol was found in every wax fraction, being isolated in crystalline form and identified in every case. We may conclude from the present available evidence that phthiocerol is a characteristic metabolic product and a constant component of the wax of the human tubercle bacillus.

The present report deals only with the separation and identification of phthiocerol. The other components of the waxes, fatty acids, carbohydrates, pigments, etc., will be reported in other publications.

After establishing the fact that phthiocerol is a constant component of the wax of the human tubercle bacillus and that it is absent from the waxes of the avian bacillus, the timothy-grass bacillus, and the leprosy bacillus, we believed that a readily identifiable substance had been found which might serve to distinguish the human type of tubercle bacillus from other members of the acid-fast group. However, it has been discovered recently in this laboratory that phthiocerol is also one of the components of the wax from the bovine tubercle bacillus. A description of the bovine bacillus wax and its cleavage products will be published in a separate report.

EXPERIMENTAL

That phthiocerol is contained in the wax fractions of the human tubercle bacillus, Strain H-37, has been shown in a former publication (1). The wax fractions used in the present investigation were prepared by Crowder, Stodola, Pangborn, and Anderson (5) and had been obtained from Strains A-10, A-12, A-13, and A-14. These strains of bacilli had been recently isolated from human cases of tuberculosis.

Since the waxes on saponification yield carbohydrate, fatty acids, pigments, and neutral substances such as phthiocerol, it was necessary to devise methods for the separation of these constituents. Thanks to previous experience, we were able to apply the following simplified procedure for the separation of the cleavage products and for the isolation of phthiocerol.

The crude chloroform-soluble wax from Strain A-10, 57.2 gm., was dissolved in 300 cc. of warm benzene and to the solution was added a hot solution of 15 gm. of potassium hydroxide dissolved

in 300 cc. of absolute alcohol. A white, brittle, solid substance separated almost immediately and collected on the sides and bottom of the flask. After the mixture had been refluxed on a water bath for 6 hours, the clear supernatant solution was decanted and the insoluble residue was rinsed several times with a hot benzene-alcohol solution.

A water-soluble carbohydrate was isolated from the alcohol-insoluble residue, as will be described later.

The decanted solution and washings were combined and refluxed on a water bath for 72 hours. Earlier experiments (1) had shown that such prolonged treatment was necessary in order to saponify the wax completely and liberate the phthiocerol. During this part of the saponification an additional small amount of insoluble material separated from the solution and was combined with the first alcohol-insoluble residue mentioned above.

The saponified solution was diluted with water, acidified with hydrochloric acid, and extracted with ether. The aqueous solution was examined for glycerol, as will be described later. The ethereal extract was washed with water until the washings were neutral, dried over sodium sulfate, filtered, and evaporated to dryness. The residue was dissolved in 100 cc. of ether and 150 cc. of cold 95 per cent alcohol were added which caused the precipitation of a white amorphous powder. After the mixture had stood at room temperature overnight, the precipitate was filtered off, washed with alcohol, and dried *in vacuo*. The product, which represented higher hydroxy acids, was reserved for future examination.

The filtrate and washings were combined and the ether was removed by distillation, after which the solution was made faintly alkaline to phenolphthalein by addition of alcoholic potassium hydroxide. To the hot solution was added an excess of lead acetate dissolved in hot alcohol and the solution was allowed to stand overnight. The precipitated lead salt was filtered off, washed thoroughly with cold alcohol, and decomposed in the usual manner, yielding a mixture of white solid fatty acids.

The filtrate and washings from the lead salt mentioned above were combined and evaporated nearly to dryness *in vacuo*. The residue was mixed with dilute acetic acid and extracted with ether, and the aqueous layer was discarded. The ethereal solu-

tion was washed with water until the washings were neutral and afterwards it was twice extracted with dilute sodium hydroxide for the removal of fatty acids which had not been precipitated as lead salts.

The alkaline washings were acidified and the fatty acids were isolated in the usual manner. A small amount of dark colored fatty acid was obtained, which was liquid at room temperature. In addition to fatty acids this fraction also contained traces of the pigment phthiocol. The separation of phthiocol from this mixture and the quantitative estimation of this pigment will be described in another publication.

Isolation of Phthiocerol

The ethereal solution, after the alkaline extraction mentioned above, was washed with water, dried over sodium sulfate, filtered, and evaporated to dryness. The residue was a white, crystalline material from which pure phthiocerol was obtained by crystallization. After three recrystallizations from ethyl acetate the substance separated in the form of rosettes of prismatic needles. The crystals melted at 72–73° and gave no depression when mixed with a sample of previously isolated phthiocerol. In chloroform solution the specific optical rotation was -4.2° .

Analysis— $C_{25}H_{42}O_2$ (540). Calculated. C 77.78, H 13.33
Found. " 77.65, " 13.36

The amounts of solvents, etc., mentioned above refer to the saponification of the wax fraction obtained from Strain A-10. In the case of the other waxes proportionally smaller volumes were used.

All the mother liquors from the crystallizations of phthiocerol from the several wax fractions were combined and concentrated. It was possible to isolate 0.85 gm. of pure phthiocerol from this material.

The final mother liquor on concentration to dryness left a reddish colored residue which weighed 2.9 gm. The material was a soft solid, much more soluble than phthiocerol, and it did not crystallize. It is quite evident that phthiocerol is the main constituent of the neutral material of the waxes but some other substances of unknown nature are also present.

Isolation of Carbohydrate

The brittle alcohol-insoluble material which separated from the saponification mixture was dissolved in water and the solution was acidified with acetic acid. A slight amount of insoluble material was filtered off and to the filtrate was added a solution of neutral lead acetate in slight excess. The small amount of precipitate which formed was filtered off and discarded. A carbohydrate was isolated from the filtrate by means of basic lead acetate and ammonia. The lead compound was filtered, washed, and decomposed with hydrogen sulfide and again filtered, and the filtrate was concentrated *in vacuo* to a thick syrup. The latter on dehydration with absolute alcohol yielded the carbohydrate as a white powder.

The carbohydrate fractions were tested in Dr. Heidelberger's laboratory and were found to give a precipitin reaction with immune serum in dilutions up to 1:1,000,000.

All of the wax fractions from the human tubercle bacillus which have been isolated in this laboratory by extraction with chloroform have been found to contain carbohydrate. The carbohydrate is presumably combined with the fatty acids. The combination must, however, be very labile, perhaps more in the nature of a salt than as an ester, because it was noted in the present investigation that in every case most of the carbohydrate was precipitated almost immediately on addition of absolute alcoholic potassium hydroxide to a solution of the wax in benzene.

Examination for Glycerol

The acidified aqueous solution from which the ether-soluble saponification products had been extracted was neutralized with sodium hydroxide and evaporated to dryness *in vacuo*. The residue was extracted with pyridine, the insoluble portion was filtered off, and the filtrate was evaporated to dryness under reduced pressure. In every case a thick syrupy residue was obtained which gave a distinct acrolein reaction when heated with acid potassium sulfate. The material obtained from wax from Strain A-10 was benzoylated according to Einhorn and Hollandt (7) and yielded crystalline glycerol tribenzoate, m.p. 75-76°.

The results obtained would indicate that small amounts of glycerol were present in all the crude wax fractions. Whether

the glycerol was an integral part of the wax or a contaminant from the medium adhering to the bacterial cells cannot be determined from the present data. The quantities of crude glycerol obtained are recorded in Table I.

We summarize in Table I the various cleavage products obtained from the wax fractions examined. No investigation has been made of the fatty acids but they have been reserved for future work. From the liquid fatty acid fractions which were dark colored it was possible to isolate small amounts of the pig-

TABLE I

Phthiocerol and Other Cleavage Products from Crude Chloroform-Soluble Wax of Human Tubercle Bacilli

Strain of bacilli.....	A-10	A-12	A-13	A-14
	gm.	gm.	gm.	gm.
Crude wax, saponified.....	57.2	32.0	5.7	29.7
Purified carbohydrate.....	6.3	4.8	0.85	5.1
Higher hydroxy acids.....	13.7	10.6	0.85	8.1
Solid fatty acids from alcohol-insoluble lead salts.....	19.2	7.1	0.9	8.6
Liquid fatty acids from alcohol-soluble lead salts.....	3.4	1.8	1.9	2.2
Crude neutral material.....	4.8	1.6	0.1	1.3
Pure phthiocerol.....	2.7	0.8	0.035	0.6
Crude glycerol.....	1.6	0.58	0.24	0.85

ment phthiocol (8) but this part of the work will be described in another publication.

SUMMARY

The wax fractions from four strains of the human type of tubercle bacilli, Strains A-10, A-12, A-13, and A-14, recently isolated from human cases of tuberculosis, have been examined for the presence of phthiocerol.

It was possible to isolate phthiocerol in pure crystalline form from the wax fractions of all four strains of bacilli.

It is evident, therefore, that phthiocerol is one of the characteristic metabolic products of the human tubercle bacillus. So far, it has only been found in the wax fractions.

A method is described by means of which it is possible to isolate pure crystalline phthiocerol in a comparatively short time.

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THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

XLIX. THE COLORIMETRIC DETERMINATION OF PHTHIICOL*

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(Received for publication, April 27, 1937)

The pigment phthiocol was first discovered by Anderson and Newman (1) as one of the constituents of the acetone-soluble fat of the human tubercle bacillus, Strain H-37. It was established by these authors that phthiocol had the formula $C_{11}H_8O_3$ and that it was 2-methyl-3-hydroxy-1,4-naphthoquinone (2). They also succeeded in synthesizing the substance from 2-methylnaphthalene (3) and by decarboxylating 3-hydroxy-1,4-naphthoquinone-2-acetic acid (4). Other methods of synthesis have been published later by Madinaveitia (5) and by Hooker (6). The free pigment crystallizes in beautiful yellow prismatic crystals and melts at 174–175°. It is very slightly soluble in water but is easily soluble in dilute alkali, giving bright red-colored solutions.

The present report deals with the isolation and identification of phthiocol from wax fractions obtained from recently isolated strains of the human tubercle bacillus. The waxes employed in this investigation were the same as those used for the identification of the dihydric alcohol phthiocerol as described in Paper XLVIII of this series (7). It was noticed that the saponification mixtures of the waxes from Strains A-10, A-12, and A-14 showed a distinct red coloration, thus indicating the presence of phthiocol, while the wax from Strain A-13 showed no red coloration on saponification.

* The present report is a part of a cooperative investigation on tuberculosis; it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

† Holder of a National Tuberculosis Association Fellowship at Yale University, 1936–37.

The amount of phthiocol present in the waxes was very small and only in the case of the wax from Strain A-10 was it possible to isolate 3.9 mg. of the substance in pure crystalline form. The following colorimetric method was therefore developed for the purpose of determining very small amounts of phthiocol.

The method is based on the fact that phthiocol gives bright red solutions in dilute alkali and the color is quite stable. Hence by comparing in a colorimeter solutions of phthiocol in 2 per cent sodium bicarbonate with similar solutions of known concentrations the amount of phthiocol in the unknown can be estimated. By means of the colorimeter we have found that amounts of phthiocol as low as 0.4 mg. can be determined with an accuracy of ± 5 per cent. By means of the spectrophotometer amounts of phthiocol less than 0.05 mg. may be estimated.

EXPERIMENTAL

Stability of Alkaline Solutions of Phthiocol—In order to determine the stability of the red-colored alkaline solutions of phthiocol, the following observations are recorded. Aqueous solutions were prepared containing 0.108 mg. per cc. of phthiocol dissolved in 0.5, 2.0, and 5 per cent sodium bicarbonate and in 5.0 per cent sodium hydroxide. When examined in a Bausch and Lomb colorimeter, all the solutions showed the same intensity of color.

In diffused light the colored solutions remained stable for several days and when the solutions were kept in the dark there was no change after standing for 4 months. For accurate determinations, however, it is to be recommended that fresh standards be prepared from synthetic phthiocol, a product that can be synthesized readily by any of the published methods.

Stability of Acid Solutions of Phthiocol—Since the method of isolating phthiocol from a saponification mixture includes the process of esterification with methyl alcoholic hydrochloric acid, the following determination was made. Phthiocol, 10 mg., dissolved in 3 cc. of absolute methyl alcohol containing 2.5 per cent of dry hydrochloric acid, was refluxed for 3 hours. The solution was diluted with water and neutralized with potassium hydroxide and 2 per cent sodium bicarbonate solution was then added and the solution extracted with ether. The alkaline solu-

tion was again extracted with ether, after which it was diluted to 25 cc. with 2 per cent sodium bicarbonate. When compared with a standard solution, a recovery of 10.6 mg. of phthiocol was indicated. It is evident, therefore, that phthiocol is not decomposed when boiled with acid in alcoholic solution.

Determination of Phthiocol in Alkaline Solution with the Spectrophotometer¹

By means of the spectrophotometer it is possible to estimate very small amounts of phthiocol in dilute alkaline solutions. Solutions of phthiocol in 2 per cent sodium bicarbonate were examined with a Pulfrich spectrophotometer with light filter No. S-50 and it was found that the extinction coefficient was a linear function of the concentration up to 2 mg. of phthiocol per

TABLE I
Extinction Coefficients of Phthiocol

Phthiocol in 10 cc. of 2 per cent NaHCO ₃	Cell length	K
mg	cm.	
0.05	3	0.065
0.10	1	0.128
0.20	1	0.258
0.30	1	0.426

100 cc., as indicated by the observations recorded in Table I. By this procedure it is possible to estimate quantities of phthiocol less than 0.05 mg.

Isolation of Phthiocol from Wax Fractions—The wax had been saponified and the cleavage products separated as described in Paper XLVIII of this series. The solubility properties of phthiocol are such that all of the pigment becomes concentrated in that fraction which contains the alcohol-soluble lead salts of fatty acids and the neutral material. This mixture of substances was dissolved in ether and after removal of the lead the ethereal solution was extracted with dilute alkali. The alkaline extract

¹ We are indebted to Dr. G. W. Fucher, Connecticut Agricultural Experiment Station, New Haven, for assistance in the use of the Pulfrich spectrophotometer.

contained the fatty acids and the pigment, while the neutral material remained in the ethereal solution.

The red-colored alkaline extract was acidified with hydrochloric acid and extracted with ether. The ethereal extract after being washed with water was extracted with 2 per cent sodium bicarbonate solution, thus removing phthiocol together with a small amount of fatty acids. The separation of phthiocol from the fatty acids was accomplished by the method described by Crowder, Stodola, Pangborn, and Anderson (8); i.e., by converting the acids to the methyl esters by refluxing the mixture with absolute methyl alcohol containing dry hydrochloric acid. After dilution with water, the esters and the pigment were extracted with ether and the ethereal extract was washed with 2 per cent aqueous sodium bicarbonate solution for the removal of phthiocol.

TABLE II
Phthiocol in Wax Fractions of Human Tubercle Bacillus

Strain of bacillus	Amount of wax	Phthiocol
	<i>gm.</i>	<i>mg.</i>
A-10	57 2	9 3
A-12	32 0	1 4
A-13	5 7	None
A-14	29 7	2 3

The alkaline solution was reextracted with ether in every case, after which it was made up to a volume of 25 cc. The red-colored solutions were diluted when necessary until they approximately matched the standard solution containing 1 mg. of phthiocol per 10 cc. The unknown and the standard solutions were then compared in the colorimeter and the amount of phthiocol in the unknown solution was calculated. By this means the amounts of phthiocol listed in Table II were estimated in the wax fractions after saponification.

Isolation of Phthiocol from Wax from Strain A-10—The wax from Strain A-10 contained the largest amount of phthiocol; hence an attempt was made to isolate the pigment in pure form from this fraction. The 2 per cent sodium bicarbonate solution which had been used for the determination recorded in Table II was acidified and extracted with ether and yielded on evaporation

of the solvent 11.1 mg. of residue which contained yellow crystals. Owing to the presence of slight impurities the substance could not be purified by crystallization and the material was therefore distilled with steam. The distillate, which contained some yellow crystalline phthiocol, was extracted with ether. On evaporation of the solvent a yellow crystalline residue was obtained which weighed 9.9 mg., an amount in agreement with the value of the colorimetric determination. After three crystallizations from 50 per cent methyl alcohol, 3.9 mg. of yellow prismatic crystals were obtained. The melting point, 174–175°, was that of pure phthiocol and there was no depression of the melting point when mixed with synthetic phthiocol, m.p. 174–175°.

SUMMARY

A colorimetric method is described by means of which it is possible to estimate about 0.4 mg. of phthiocol.

The method has been applied to the determination of phthiocol in the wax fractions from recently isolated strains of the human tubercle bacillus.

By means of the spectrophotometer, it is possible to estimate less than 0.05 mg. of phthiocol in 2 per cent sodium bicarbonate solutions.

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THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

L. THE OCCURRENCE OF PHTHIOCEROL IN THE WAX OF THE BOVINE TUBERCLE BACILLUS*

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Recent investigations in this Laboratory have shown that the wax fractions of the human tubercle bacillus yield on saponification an easily identifiable crystalline, dihydric alcohol which was named phthiocerol and which corresponds to the formula $C_{35}H_{72}O_2$ or $C_{36}H_{74}O_2$ (1). In addition to phthiocerol, the waxes contain several fatty acids, principally hydroxy acids of very high molecular weight (2), and a specific polysaccharide (3).

It is a significant fact that phthiocerol is not a constituent of the waxes from other acid-fast bacteria such as the timothy-grass bacillus (4), the leprosy bacillus (5), or the avian tubercle bacillus (6), the waxes from these organisms containing in place of phthiocerol the two higher optically active alcohols, *d*-eicosanol-2, $C_{20}H_{42}O$, and *d*-octadecanol-2, $C_{18}H_{38}O$. It seemed, therefore, a matter of some importance to be able to differentiate sharply between the human tubercle bacillus and other acid-fast bacilli by means of the well defined crystalline alcohols which are present in the waxes.

It will be shown in the present report that the wax from the bovine tubercle bacillus contains the same higher alcohol, namely phthiocerol, as is present in the human type of tubercle bacillus. The meaning of this observation is not clear at the present time but it would indicate a closer relationship between the human and bovine tubercle bacilli than between the avian, timothy, and leprosy bacilli. Although the waxes of the human and bovine tubercle bacilli contain the same alcohol, phthiocerol, the polysaccharides in the two waxes are entirely different and distinct.

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† Holder of a National Tuberculosis Association Graduate Fellowship at Yale University, 1936-37.

The present report deals only with the isolation and identification of phthiocerol. The other components of the bovine bacillus wax, fatty acids and polysaccharide, will be described in a later publication.

EXPERIMENTAL

The wax used in this investigation was the chloroform-soluble material isolated by Anderson and Roberts (7) as described in a former publication. The wax was purified by precipitation from ethereal solution by addition of methyl alcohol. After five precipitations a nearly white, granular powder was obtained. The following constants were determined: m.p. unsharp 47–54°, iodine number (Hanus) 3.2, $[\alpha]_D$ in benzene +15.6°. The substance contained P 0.30 and N 0.15 per cent.

The purified wax was saponified and the cleavage products were separated essentially as described by Reeves and Anderson (6) for the avian tubercle bacillus wax. The yield of neutral material from 60.3 gm. of the wax was 2.1 gm. or about 3.5 per cent. The neutral material was a white crystalline solid.

Purification of Neutral Material. Isolation of Phthiocerol—The neutral material was crystallized three times from ethyl acetate and it separated in the form of aggregates of fine prismatic crystals. The crystals melted at 73–74°, solidified at 72°, and remelted at 73–74°. When the substance was mixed with a previously isolated sample of phthiocerol, the melting point was not depressed. Eight further recrystallizations from ethyl acetate caused no change in properties.

Rotation—0.4183 gm. of substance dissolved in chloroform and made up to 10 cc. gave in a 1 dm. tube $\alpha = -0.17^\circ$; hence $[\alpha]_D^{20} = -4.06^\circ$.

Analysis—3.881 mg., 5.784 mg. substance gave 4.686 mg., 6.936 mg. H_2O and 11.025 mg., 16.450 mg. CO_2

$C_{33}H_{73}O_2$ (540). Calculated. C 77.78, H 13.33

$C_{33}H_{74}O_2$ (554). " " 77.98, " 13.35

Found. " 77.48, 77.57, " 13.42, 13.32

Hydroxyl Determination— $C_{33}H_{73}O_2$ (540). Calculated. $(OH)_2$ 6.29

$C_{33}H_{74}O_2$ (554). " " 6.13

Found. " " 6.09

Methoxyl Determination— $C_{33}H_{73}O_2$ (540). Calculated. OCH_3 5.74

$C_{33}H_{74}O_2$ (554). " " 5.59

Found. " 4.63, 4.30

Phthiocerol does not yield any well characterized derivatives. The acetyl derivative is a low melting solid which could not be obtained in crystalline form (1). The benzoate, the phenylurethane, *p*-nitrophenylurethane, and diethylacetylurethane were prepared in the course of the present study but they were all non-crystalline. The phenylurethane derivative was the most promising but it could only be obtained as a non-crystalline powder, m.p. 78–82°.

Analysis—

$C_{15}H_{33}O_2N_2$ (778).	Calculated.	C 75.51, H 10.61, N 3.60, OCH ₃ 3.98
$C_{16}H_{34}O_2N_2$ (792).	“	“ 75.69, “ 10.68, “ 3.53, “ 3.91
	Found.	“ 75.72, 75.84, H 10.44, 10.61, N 3.30, 3.35, OCH ₃ 3.04

It will be noted that in all the analyses for methoxyl the values obtained were slightly lower than the calculated value. The reason for the low methoxyl values is not apparent. In all other respects, in solubility, crystal form, melting point, optical rotation, and composition, the alcohol isolated from the bovine tubercle bacillus wax was identical with phthiocerol.

SUMMARY

A dihydric, monomethoxy alcohol of the formula $C_{23}H_{47}O_2$ or $C_{24}H_{49}O_2$ has been isolated from the bovine tubercle bacillus wax. The properties of this alcohol are identical with those of phthiocerol, the characteristic alcohol of the human tubercle bacillus wax.

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A TITRIMETRIC METHOD FOR THE QUANTITATIVE ESTIMATION OF LEAD IN BIOLOGICAL MATERIALS

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The announcement by Fischer in 1929 (1) of the remarkable affinity of dithizone (diphenylthiocarbazone) solutions for lead has stimulated many laboratories to search for practical micro-methods that could be applied to the determination of lead in biological materials. The theory and the application of such methods have been adequately described by Wilkins, Willoughby, Kraemer, and Smith (2), Ross and Lucas (3), and more recently by Clifford and Wichmann (4) and will not be treated further here.

The authors have had the opportunity to apply the various dithizone methods to a large variety of materials. Most of the published techniques have been critically investigated and this paper will present what is in our opinion the most satisfactory extraction procedure together with a new titration that eliminates the necessity of investing in expensive photometric equipment.

Interference by Bismuth and Tin—The red color produced by the reaction between solutions of dithizone in chloroform and those of a heavy metal in alkaline cyanide solution is not entirely specific for lead (5). Bismuth and stannous tin react with dithizone in a similar fashion and must be eliminated at some point in the analytical procedure. Bismuth may be present in biological specimens as a result of previous medication and stannous tin is not an uncommon constituent of the normal diet.

Fischer and Leopoldi (6), Winter *et al.* (7), and Tompsett and Anderson (8) have made use of the fact that bismuth may be separated from lead by extracting the dithizone mixture with

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solutions of alkaline cyanide. Clifford and Wichmann (4) have objected to this procedure on the grounds that the alkaline cyanide extracted some of the lead along with the bismuth. Our investigations have shown us that the amount of lead extracted by washing with potassium cyanide solution varied not only with the concentration of the dithizone present but also with the concentration of the potassium cyanide used. Thus the 1 per cent solutions of potassium cyanide caused an appreciable loss of lead (5 to 10 per cent), but if a 0.5 per cent solution is used, the loss of lead is negligible, if the extraction is properly conducted. Where comparatively large amounts of bismuth are present (25 times the quantity of lead or more), it is advisable to use the procedure proposed by Willoughby and associates (9) and extract the weakly acidified solution with an excess of dithizone. We suggest, however, that the aqueous solution containing the lead and bismuth be adjusted to pH between 3.0 and 3.5, instead of pH 2 as recommended by Willoughby *et al.*, in order to obtain a more satisfactory separation. Since bismuth will react with dithizone to give a brown color to the chloroform, a large excess of bismuth is easy to notice.

It is not possible to make an efficient separation of stannous tin from lead by extracting an acidified mixture with dithizone, because the optimum pH for the reaction between stannous tin and dithizone is close to neutrality. Fortunately, stannic tin does not react with dithizone, and since the process of ashing converts the stannous tin to stannic tin, the former does not interfere. Small amounts of stannous tin can be separated from a chloroform solution of lead dithizonate by shaking with 0.5 per cent potassium cyanide. Any traces of tin which have not been oxidized or which have reverted to the stannous state are removed from the mixture by the cyanide solution.

Interference by Calcium Phosphate—The presence of large amounts of calcium phosphate, as in the analyses of bones, presents a special problem, if one wishes to avoid using a sulfide precipitation. Winter and collaborators (7) have suggested a preliminary separation of the lead phosphate which they claim precipitates at a lower pH than calcium phosphate. Similarly, in the analyses of urine, Ross and Lucas (3) precipitate the calcium as the oxalate at pH 4.5, a procedure which entrains the lead and leaves most of the phosphate in solution.

It has long been known that citrates exert a solvent action on the phosphates of calcium (10). In 1881, Terreil (11) showed that 7 gm. of calcium phosphate were dissolved by a solution of 100 gm. of citric acid which had been neutralized with ammonia. In analytical work it is not practical to use such large amounts of citric acid. Large concentrations of citric acid not only increase the specific gravity of the aqueous solution, thus slowing the chloroform separations, but also reduce the affinity of dithizone for lead so that large excesses of dithizone must be used to insure complete extraction. These disadvantages may be avoided, however, if proper adjustments are made between the volume of solution and the amounts of calcium phosphate and citrate used. Thus it was found that the lead in an aliquot equivalent to not more than 1.5 gm. of dry bone could readily be extracted from 350 cc. of solution at pH 8.0 containing 15 gm. of sodium citrate. A "wet digestion" with sulfuric, nitric, and perchloric acids was used during our preliminary work on lead methods, but was soon abandoned because of the large amounts of lead introduced by this acid mixture. Furthermore, the use of sulfuric acid complicated the analysis of materials high in calcium. Better results were obtained when "dry ashing" at a temperature below 500° was used. In order to facilitate the preliminary charring of the biological materials, an overhead heater was devised by Nims and Horwitt (12), which greatly shortened the time required for an analysis. By application of radiant heat from above, a sample can be dehydrated and charred without danger of foaming or spattering. The resulting product may be placed directly into a muffle furnace at 475°.

A source of error in the analysis of lead may be found in the type of dish used for ashing when the quantities of ash are small. When 5 cc. portions of a lead nitrate solution containing 0.01 mg. of lead per cc. were evaporated in a porcelain dish and heated at 450° for 2 hours, less than 80 per cent of the original lead was recovered by extraction with hot 20 per cent hydrochloric acid. Two extractions with alkaline citrate and two more with 10 per cent potassium cyanide accounted for another 15 per cent of the original lead. When the same experiment was repeated with silica dishes, the hydrochloric acid alone extracted 97 per cent or more of the added lead.

Interference by Iron—Dithizone solutions are oxidized by ferric

iron in the presence of cyanide and special precautions must be taken when blood or other materials containing much iron are analyzed. Wilkins and associates (2) resorted to a preliminary dithizone extraction in which the partial destruction of this reagent was not important. Tompsett and Anderson (8) also used a preliminary extraction, except that in their case sodium diethyldithiocarbamate was used instead of dithizone. A simple procedure was suggested by Fischer and Leopoldi (13) who add hydroxylamine hydrochloride to prevent the oxidation of dithizone. This treatment is effective if the amounts of iron present are small.

Since ferric iron in the concentrations encountered does not oxidize dithizone in the absence of cyanide, Wichmann *et al.* (14) have suggested that less cyanide be used. Similarly, Cheftel and Pigeaud (15) reduced the amount of cyanide used and claimed that iron did not interfere with the extraction of lead under these conditions. In our own laboratory the best results were obtained when both hydroxylamine hydrochloride and reduced amounts of potassium cyanide were employed; this procedure permitted the analysis of larger amounts of blood and gave good results.

Principle of Titration—The method described below differs from other dithizone techniques in that it is not necessary to standardize the dithizone solution. The final titration is carried out directly with a dilute lead solution, thus eliminating the necessity for special precautions in the handling of the dithizone. The lead is separated from a given solution by means of dithizone and the resulting lead-dithizone complex is then isolated. The latter is freed of lead by washing with acid. The chloroform solution of dithizone remaining is mixed with some dilute cyanide solution which removes most of the dithizone from the chloroform, imparting a brown color to the aqueous layer. A lead solution is added from a burette to this mixture until all the dithizone has been reconverted to lead dithizonate, as indicated by (1) the disappearance of the brown color in the aqueous layer, and (2) the absence of a red color when the aqueous layer is mixed with chloroform and additional lead solution.

Apparatus—All glassware should, whenever possible, be made of Pyrex glass. Silica dishes are recommended for the ashing

procedures. The cleansing of the separatory funnels before each analysis is especially important. Washing with hot dilute nitric acid followed by a thorough rinsing with water redistilled from an all-Pyrex still is usually sufficient for the separatory funnels, but the dishes in which materials have been ashed should be cleansed with the aid of a warm solution of alkali as well.

An overhead heater of some sort is recommended for preparing biological samples (12). Such a device not only shortens the time required to complete a determination but also affords protection from laboratory dust during the diminished period of handling. The muffle furnace used should preferably be equipped with a stainless steel sleeve to protect the samples from contamination by the brittle parts of the oven.

Reagents—The water should be distilled from an all-Pyrex still and all reagents should be stored in Pyrex containers.

1. Potassium cyanide solution. 10 gm. in 100 cc. prepared daily.

2. Hydrochloric acid, 20 per cent. Concentrated acid (sp. gr. 1.19) mixed with an equal volume of water and the mixture distilled from the all-Pyrex still.

3. Hydrochloric acid, 0.5 per cent. Prepared by diluting 25 cc. of Reagent 2 to 1 liter.

4. Chloroform, U.S.P.

5. Hydroxylamine hydrochloride solution, 25 per cent.

6. Dithizone solution. Dissolve 40 mg. of diphenylthiocarbazone in 400 cc. of chloroform and filter into a 500 cc. Pyrex separatory funnel. Add 50 cc. of water containing 2 cc. of 25 per cent hydroxylamine hydrochloride solution and shake. Keep in a cool dark place and withdraw the chloroform solution as needed. The acid aqueous layer not only prevents the oxidation of the dithizone but also extracts any lead which might be present. Further purification was not found necessary for the titrimetric method to be described below.

7. Potassium cyanide solution, 0.5 per cent. Prepared daily by diluting 25 cc. of Reagent 1 to 500 cc. It is important that this solution be lead-free. To insure this, place 100 cc. of Reagent 1 in a separatory funnel and extract with 2 cc. of chloroform containing 2 drops of dithizone solution. If a pink color appears in the chloroform layer, withdraw it and repeat the extraction

until the chloroform layer is colorless. The slight excess of dithizone which remains in the 10 per cent potassium cyanide is not significant, since the amounts which remain after dilution to form the 0.5 per cent solution are not detectable.

8. Nitric acid. Redistil the concentrated reagent.

9. Ammonium hydroxide. Distil the concentrated reagent into cold redistilled water.

10. Standard lead solutions. Dissolve 1.599 gm. of recrystallized lead nitrate (or the equivalent of lead acetate) with the aid of 1 cc. of nitric acid and dilute to 100 cc. This solution which contains 10 mg. of lead per cc. is quite stable. By diluting 10 cc. of this solution to 100 cc. and then in turn diluting 10 cc. of the latter to 1 liter, a solution containing 0.01 mg. of lead per cc. is prepared. This is stable in Pyrex glass containers for at least 5 days.

11. Sodium citrate, 20 per cent. To 800 cc. of this solution add 8 cc. of 10 per cent potassium cyanide and extract in a 1 liter separatory funnel with 15 cc. portions of dithizone solution until the citrate mixture is free of lead. Wash twice with 25 cc. portions of chloroform, acidify with 4 cc. of 20 per cent hydrochloric acid, and complete the extraction of the excess dithizone with 20 cc. portions of chloroform.

Procedure

Blood—A 10 cc. sample in a 50 cc. silica evaporating dish is dried and completely charred beneath a radiant heater. This is accomplished in approximately 45 minutes. Transfer to a muffle adjusted to a temperature of about 475°. After 2 hours, remove from the muffle, moisten with 2 cc. of nitric acid, and place the dish beneath the radiant heater until the reaction has ceased and the material is free of excess acid. This requires approximately 30 minutes. Return to the muffle for about half an hour to complete the oxidation.

Place the dish on a hot-plate, carefully add 15 cc. of 20 per cent hydrochloric acid, and heat until the ash is dissolved. Wash the contents into a 125 cc. separatory funnel with about 20 cc. of hot water. Add 10 cc. of 20 per cent sodium citrate and 3 cc. of ammonium hydroxide to the silica dish, mix, and transfer to the separatory funnel with enough water to make a total volume

of about 75 cc. Cool, add 1 cc. of hydroxylamine hydrochloride, 1 drop of phenol red, and bring to pH 8.0 with ammonium hydroxide delivered from a Pyrex burette. Cool, add drop by drop, shaking between additions, 0.5 cc. of 10 per cent potassium cyanide, and immediately extract with 0.5 cc. of dithizone solution and 4 cc. of chloroform. If, after shaking, the chloroform layer does not contain a noticeable excess of uncombined dithizone, add 0.2 cc. portions of dithizone solution, shaking between additions, until the green excess becomes evident. Remove the chloroform phase to another separatory funnel and repeat the extraction of the aqueous phase twice with 0.2 cc. portions of dithizone in 2 cc. of chloroform. To the combined chloroform solutions add an amount of 0.5 per cent potassium cyanide equal to 1.5 times the volume of the chloroform solution and shake for 10 seconds. Withdraw the chloroform layer to another separatory funnel and wash the aqueous cyanide solution with 1 cc. of chloroform. Combine the chloroform solutions and again extract with 1.5 volumes of 0.5 per cent potassium cyanide solution. Any lead which may have dissolved in the aqueous phase is removed by extraction with 2 cc. of chloroform.

The extraction with cyanide solution described above removes the uncombined dithizone unless a very large excess has been used, in which case the extraction with 0.5 per cent potassium cyanide is continued until the absence of color in the aqueous layer indicates that the dithizone excess has been removed. The lead is separated from the red dithizone complex by shaking for 15 seconds with 2 volumes of 0.5 per cent hydrochloric acid. Withdraw the green chloroform layer and then extract the acid aqueous solution with 1 cc. of chloroform to recover the last traces of dithizone. Combine the chloroform fractions.

Titration—Add to the dithizone solution 0.5 volume of 0.5 per cent potassium cyanide and shake. Most of the dithizone goes into the aqueous layer, giving that mixture a brown color. Add the standard lead solution (0.01 mg. per cc.) from a burette a drop at a time, shaking between additions, until only a very faint color remains in the aqueous phase. This is evidence that practically all of the dithizone has combined with lead and gone into the chloroform layer. Discard the red chloroform phase and wash the aqueous layer with chloroform, 2 cc. at a time, until the chloro-

form layer remains colorless after shaking. (In order to prevent any loss of uncombined dithizone the color in the cyanide solution should not be greater than that color which 0.2 cc. of Reagent 6 will impart to 10 cc. of 0.5 per cent potassium cyanide solution.) Add a drop or two of the lead solution and shake for 5 seconds. Withdraw the pink chloroform solution and continue the extraction with 2 cc. portions of chloroform plus a drop or two of lead solution until further addition of lead gives no pink color to the chloroform solution after shaking. The end-point is a slight pink in the chloroform solution; extraction with 1 more drop results in a colorless solution. In order to facilitate the titration, a solution of the lead-dithizone complex containing a small amount (1 or 2 drops) of the lead solution in 2 cc. of chloroform is kept for comparison. When the color obtained after an addition of lead solution is less than that given by 1 drop of lead, the end-point has been attained. It is suggested that the beginner add 2 drops (about 0.0006 mg.) at a time until his eyes become accustomed to the change.

Urine—Measure 200 cc. of urine into a 250 cc. silica dish and ash as described above for blood, except that 5 cc. of nitric acid should be used instead of 2 cc.

Because the ash of urine is sometimes difficult to dissolve, moisten it with 15 cc. of 20 per cent hydrochloric acid and heat until almost dry. Transfer the contents to a 500 cc. separatory funnel with the aid of an additional 15 cc. of hydrochloric acid, 50 cc. of 20 per cent sodium citrate, and 5 cc. of ammonium hydroxide. Make to a volume of 250 cc. and cool. Add 1 drop of phenol red and slowly bring to pH 8.0 with ammonium hydroxide. Add 3 cc. of 10 per cent potassium cyanide; extract with an excess of dithizone using 0.5 cc. portions in 3 cc. of chloroform and proceed as with blood beginning with "To the combined chloroform solutions add. . ."

Bone—Place a known amount of dried bone in a silica dish and heat in a muffle at 475° for 2 hours. Remove, add an amount of nitric acid equivalent to 3 cc. for each 1.5 gm. of dried bone, and evaporate to dryness beneath the radiant heater. Return to the muffle for about $\frac{1}{2}$ hour.

Dissolve the contents of the silica dish, using 15 cc. of 20 per cent hydrochloric acid for each 1.5 gm. of dried bone, and transfer

an aliquot containing not more than 1.5 gm. to a 500 cc. Pyrex separatory funnel. Add 75 cc. of sodium citrate solution, 2 drops of phenol red, and enough water to make a volume of about 350 cc. Add ammonium hydroxide 1 drop at a time, shaking and cooling during the addition, until pH 8.0 has been attained. Add slowly 5 cc. of 10 per cent potassium cyanide and extract the clear solution with an excess of dithizone. Add 3 cc. of dithizone solution and 3 cc. of chloroform, and shake for 30 seconds. If the chloroform layer is not purple, add 1 cc. of dithizone solution at a time until the purple color remains after shaking, indicating that a large excess of dithizone has been used. Good results are obtained in the presence of large quantities of citrate, if a 100 per cent excess of dithizone is used at this stage. Withdraw the chloroform layer to a 125 cc. separatory funnel and extract the aqueous mixture three times with 1 cc. of dithizone solution plus 2 cc. of chloroform, 0.5 cc. of dithizone plus 2 cc. of chloroform, and 3 cc. of chloroform, respectively. Combine the chloroform solutions and extract once with 2 volumes and twice with equal volumes of 0.5 per cent potassium cyanide. Remove the lead from the lead-dithizone complex with 2 volumes of 0.5 per cent hydrochloric acid and titrate the resulting dithizone solution as described under blood beginning, "Add to the dithizone solution 0.5 volume. . ."

Blank Determination—A separate blank is run for each type of material analyzed. It is also important that an amount of lead approximately equivalent to that which one is likely to obtain from the material be added to the reagents when the blank is estimated in order that the blank determination may serve as a daily check on the technique of the analyst. This permits the use of as much dithizone as in the regular procedure and may bring out errors of manipulation that might not otherwise be caught. Thus, if 0.02 mg. of lead is added to the reagents and the final titration shows that 0.0223 mg. was present, the blank for reagents for a determination between 0.01 mg. and 0.03 mg. of lead would be 0.0023 mg.

Notes on Procedure

1. A good grade of white vaseline is used for stop-cocks.
2. Care must be taken during the separation of the liquid

phases that no drops of the chloroform layer remain on the surface of the aqueous layer or adhere to the sides of the funnel.

3. If a precipitate should appear in the alkalized solution of the ash just prior to the extraction of the lead with dithizone, redissolve the suspension with hydrochloric acid and add more sodium citrate before again alkalizing.

4. It is sometimes more satisfactory to extract the aqueous layer with more chloroform rather than wait for the last traces of chloroform to separate out.

5. A small chloroform trap should always be maintained in the separatory funnel, rather than to attempt a complete separation.

EXPERIMENTAL

Accuracy of Fundamental Titration—Inasmuch as the final measurement in this method involves a determination of an unknown quantity of dithizone in terms of its combination with lead, it is necessary to prove the accuracy of such a titration. This was done as follows:

Measured quantities of a dilute solution of dithizone were transferred to a separatory funnel, diluted with chloroform to 15 cc., mixed with 10 cc. of 0.5 per cent potassium cyanide, and titrated with lead nitrate solution. The average time for each titration was approximately 5 minutes. The results as given in Table I indicate that the sensitivity of the titration is about 0.0002 mg.

Accuracy of Method Applied to Biological Materials—Representative results for the recovery of different amounts of lead added to blood, urine, and bones are given in Table II. The amounts added correspond to the lead which may be found in normal and pathological states. The accuracy of the method for blood is about 10 per cent, for urine and bones about 3 per cent.

A large number of foods and diets have been analyzed for lead during the past year, and we find it preferable not to use a definite procedure in these cases because of the varying amounts of iron and calcium in different products. Instead, the method is modified to suit the material. Thus, animal rations which may be high in calcium are treated by the technique described under bone. Should the material contain a considerable amount of iron, care is taken to use some hydroxylamine hydrochloride and a minimum of the 10 per cent potassium cyanide solution before extraction.

The analyst is safe, whatever procedure is used, provided a blank determination accomplished in a similar manner gives a good recovery.

TABLE I
Titration of Dithizone with Lead Nitrate

Dithizone	PbNO ₃ (0.01 mg. per cc.)	Lead nitrate used for each cc. of dithizone
cc.	cc.	cc.
1	0.52	0.52
3	1.56	0.52
3	1.54	0.51
6	3.14	0.52
6	3.13	0.52
10	5.18	0.52
10	5.18	0.52

TABLE II
Recovery of Lead from Blood, Urine, and Bones

Material	Lead in material	Lead added	Total lead recovered	Added lead recovered	Error	Recovery
	mg.	mg.	mg.	mg.	mg.	per cent
10 cc. beef blood	0.0016	0.0010	0.0025	0.0009	0.0001	90
		0.0020	0.0036	0.0020	0.0000	100
		0.0030	0.0047	0.0031	0.0001	103
		0.0040	0.0060	0.0044	0.0004	110
		0.0050	0.0066	0.0050	0.0000	100
200 cc. human urine	0.0060	0.0100	0.0163	0.0103	0.0003	103
		0.0200	0.0285	0.0205	0.0005	103
		0.0300	0.0358	0.0298	0.0002	99
		0.0400	0.0455	0.0395	0.0005	99
		0.0500	0.0560	0.0500	0.0000	100
1.5 gm. bone ash*	0.0128	0.0100	0.0230	0.0102	0.0002	102
		0.0300	0.0429	0.0301	0.0001	100
		0.0500	0.0637	0.0509	0.0009	102
		0.1000	0.1128	0.1000	0.0000	100
		0.1500	0.1618	0.1490	0.0010	99

* Aliquots of an acid solution of bone ash.

DISCUSSION

The dithizone methods published to date depend upon the standardization, at some time or other, of the dithizone solution used. Since the ultimate standard is a lead solution, a method

which does not depend on the concentration of the dithizone and makes direct use of a lead standard instead has many advantages. Furthermore, since the dithizone complexes fade on standing, a method in which use of a 10 cc. burette is substituted for a colorimetric procedure should prove more accurate. Another advantage of the method is that the amounts of bismuth and tin which are present in biological specimens are eliminated by the regular procedure.

Each determination of the blank serves not only as a daily check on the skill of the operator, but also on the efficacy of the method. The short period of heating described is important in the prevention of loss of lead; therefore, care should be exercised not to prolong the ashing procedure.

For routine analyses of foods in a control laboratory, it is probably sufficient to titrate to the disappearance of the brown color in the potassium cyanide solution.

SUMMARY

A quantitative method for the determination of lead based on a new titrimetric procedure is described. Results of the application of this method to biological materials are reported.

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THE ISOLATION OF PREGNANE-3,17,20-TRIOL FROM THE URINE OF WOMEN SHOWING THE ADRENO-GENITAL SYNDROME

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During the past year chemical studies on the urines of men and women showing the adreno-genital syndrome have yielded results of considerable interest. Callow (1) has isolated large amounts of dehydroisoandrosterone from the urine of a young girl suffering from an adrenal tumor, and who showed symptoms of virilism. Burrows, Cook, and Warren (2) have isolated a number of new compounds from the urine of a man with an adrenal tumor who showed symptoms of feminism, but only one of these, an androstadiene-17-one, has been chemically characterized.

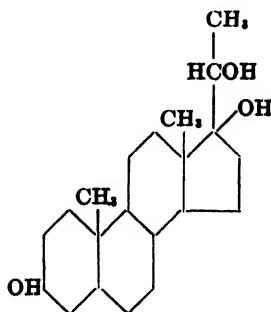
The present authors have been fortunate in being enabled to collaborate with Mr. L. R. Broster, of the Charing Cross Hospital, London, in a chemical examination of urine specimens obtained from two young women who showed the adreno-genital syndrome. A preliminary account of some of the results obtained has been published previously (Broster and Vines (3)).

The neutral, ether-soluble fractions obtained from the concentrated but unhydrolyzed preoperative urines yielded considerable quantities of a white crystalline solid which after frequent crystallization melted at 243–244°, and gave analytical figures closely corresponding to the composition $C_{21}H_{36}O_3$. This substance was not found in a urine sample collected from one of the women after removal of the enlarged adrenal; neither has it been found in urine samples collected from normal men and normal pregnant and non-pregnant women. Its presence in urine would therefore appear to be associated with the adreno-genital syndrome.

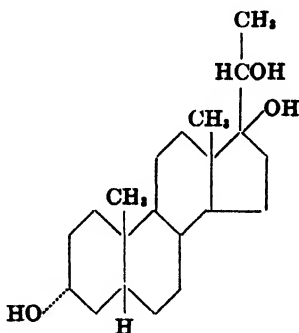
By acetylation of the new compound an acetate of the composi-

tion $C_{25}H_{40}O_3$ was obtained, indicating that 2 of the 3 oxygen atoms in the original compound were present as esterifiable hydroxyl groups. This acetate was found to react with magnesium methyl iodide with the evolution of gas. It therefore appeared probable that the 3rd oxygen atom of the parent substance was present in a non-reactive tertiary hydroxyl group.

Treatment of the new triol with the Rosenmund-Kuhnhenh bromine-pyridine sulfate reagent showed that it was fully saturated. It therefore clearly possessed a tetracyclic structure, and in view of the fact that the work of Reichstein and of Kendall *et al.* (4-6) has shown the presence of a number of steroids in the adrenal cortex, many of which contain 21 carbon atoms, it seemed reasonable to suppose that the new triol possessed a steroid skeleton of the pregnane or allopregnane type. Reichstein ((4) p. 402) and Kendall *et al.* (5, 6) have suggested that certain of the adrenal steroids possess a tertiary hydroxyl at C_{17} . In view of this suggestion the working hypothesis was adopted that the new triol had the structure (I). In order to test this hypothesis, the



I



II

triol was oxidized with lead tetraacetate by the method of Criegee (7). Titration of the excess tetraacetate showed the presence of one glycol grouping per molecule, and from the products of the oxidation were isolated the following: (A) acetaldehyde (as its 2,4-dinitrophenylhydrazone); a substance (B) that reacted with the Zimmermann (8) ketone reagent and which yielded a crystalline semicarbazone melting at 264-265°. The latter gave carbon and hydrogen analyses close to those required for a semicarbazone

of a hydroxy ketone of the formula $C_{19}H_{30}O_2$; and small amounts of a white crystalline solid (C) melting at $233-234^\circ$, which did not react with the Zimmermann reagent and which analyzed well for the formula $C_{21}H_{34}O_2$.

The formation of acetaldehyde and of the ketone (B) by the lead tetraacetate oxidation proved the correctness of the suggested structure of the side chain of the triol. On the assumption that the triol possessed a steroid carbon skeleton with a hydroxyl group at the C_3 position, then it followed that (B) was either androsterone, isoandrosterone, 3-epihydroxyetiocholane-17-one, or 3-hydroxyetiocholane-17-one. Since the original triol was not precipitated by digitonin under conditions satisfactory for the precipitation of cholesterol and isoandrosterone, the second and fourth of these possibilities seemed to be excluded. Furthermore, it was shown that the semicarbazone of (B) was not identical with either the semicarbazone of androsterone (m.p. $304-305^\circ$) or of isoandrosterone (m.p. $288-289^\circ$). It seemed probable, therefore, that (B) was 3-epihydroxyetiocholane-17-one. The latter compound was accordingly prepared by the oxidation of epicoprosterol acetate by the method of Ruzicka *et al.* (9, 10). The melting point of its semicarbazone was not depressed by admixture with the semicarbazone of (B). The substance (B) was therefore proved to be 3-epihydroxyetiocholane-17-one, and the triol to be pregnane-3,17,20-triol (structure (II)).

The presence of small amounts of the substance (C) in the products of the oxidation of the triol was at first somewhat puzzling, as it was difficult to understand how a non-ketonic product could be formed by the oxidation of the triol with lead tetraacetate. However, in view of the small amounts of (C) found in the oxidation product and in view of the fact that, owing to shortage of material, the triol specimens used for the lead tetraacetate oxidation were not of such a high degree of purity as that of the analytical specimen, it seems certain that (C) was originally present as an impurity in the triol. Subsequently it was found that a mixture of equal parts of (C) and the analytically pure triol showed a melting point intermediate between those of the two components of the mixture. It is not surprising therefore that the presence of an appreciable quantity of (C) in the less carefully purified triol was not revealed by a melting point determination.

Although the quantities of (C) available for examination were extremely small, it was finally identified satisfactorily by crystalline form and mixed melting points as pregnanediol.

EXPERIMENTAL

Isolation of the New Substance—The following urine specimens were examined: (a) 6 liters collected from J. K. (preoperative), (b) 2.5 liters from J. K. (postoperative), and (c) 6 liters from M. M. (preoperative). Each urine specimen was evaporated to a thick paste by Dr. Jocelyn Patterson at the Charing Cross Hospital, despatched to Toronto, and treated in the following manner. After dilution with 500 ml. of water, the concentrated urine was extracted four times with ether. The ethereal extract was washed three times with 5 per cent sodium carbonate solution, three times with 0.1 N sodium hydroxide, three times with water, and then evaporated to dryness. After the residue was washed with a small volume of cold benzene, a slightly colored, partially crystalline solid remained. This was decolorized in ethanolic solution with charcoal and finally washed with a small volume of cold acetone. This crude material melted at about 200–205°. The yields obtained from the three urine specimens examined were (a) 242 mg., (b) nil, (c) 95 mg.

The two batches of crude crystalline material were combined for purification. After three recrystallizations from benzene-methanol, 23.2 mg. of well formed stout needles with a constant melting point of 243–244° were obtained.

The aqueous phase after the initial ether extraction was in each case adjusted to pH 1.5 with hydrochloric acid, boiled for 2 hours, and then again extracted with ether. No further quantities of the new substance were isolated from such ether extracts obtained after acid hydrolysis.

Properties of the New Substance—The substance was moderately soluble in methanol, ethanol, and dioxane. It was almost insoluble in water, cold acetone, and benzene. Nitrogen, sulfur, and halogens were absent. The xanthoproteic and Millon tests were negative, and the substance was not precipitated from 90 per cent ethanolic solution by digitonin.

3.689 mg. gave 10.150 mg. CO₂ and 3.520 mg. H₂O

C₂₁H₃₄O₃. Calculated. C 74.93, H 10.79

Found. " 75.06, " 10.68

An iodine number determination on 2.86 mg. with the Rosenmund-Kuhnhehn bromine-pyridine sulfate reagent gave a value of 10.1. Since the theoretical iodine number of a substance of the composition $C_{21}H_{38}O_2$ with one double bond is 75.5, it was apparent that the substance was fully saturated.

10.1 mg. were acetylated by heating to 100° for 2 hours with 2 ml. of acetic anhydride and 1 drop of pyridine. After one recrystallization of the crude acetate from 50 per cent ethanol, crystals melting sharply at 136.5° were obtained.

3.926 mg. gave 10.320 mg. CO_2 and 3.360 mg. H_2O

$C_{21}H_{38}O_2$. Calculated. C 71.38, H 9.59

Found. " 71.74, " 9.58

On treatment of the purified acetate with an ethereal solution of methyl magnesium iodide, a gas was evolved. In a parallel experiment no gas was evolved by pregnanediol acetate.

Oxidation of the Triol with Lead Tetraacetate. Titration of Glycol Groups—Four oxidations were conducted on small amounts of triol recovered from the benzene-methanol mother liquors of the analytical sample. The following procedure was adopted. 10 to 25 mg. of the triol were allowed to react at room temperature in a small flask for 20 hours with 5 ml. of an approximately 0.1 N solution of lead tetraacetate in purified glacial acetic acid. During the whole period of reaction a slow stream of dry nitrogen was blown over the surface of the mixture and then bubbled through a solution of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid. Control experiments without the triol were simultaneously conducted in each instance.

At the end of the reaction, 10 ml. of the potassium iodide-sodium acetate solution (Criegee) were added to each flask and the iodine liberated titrated with 0.1 N sodium thiosulfate solution. The results obtained in four successive experiments on 21.1, 13.7, 23.2, and 10.1 mg. of triol were respectively 0.83, 0.79, 1.02, and 1.21 glycol groups per molecule.

Identification of the Volatile Oxidation Product—In each experiment a copious orange precipitate was formed in the dinitrophenylhydrazine solution. In the first experiment this precipitate was filtered off and warmed with a small volume of ethanolic hydrochloric acid, when the characteristic apple-like odor of acetaldehyde was at once detected. In the second and third ex-

periments, the precipitates were filtered off, washed thoroughly with water, dried, and crystallized from 95 per cent ethanol. Products melting at 150–152° and 152–155° were obtained. On admixture of each with authentic acetaldehyde-2,4-dinitrophenyl-hydrazone the melting points were 148–151° and 153–156° respectively. The identification of the volatile oxidation product as acetaldehyde was therefore considered to be complete.

Identification of the Non-Volatile Oxidation Products—The reaction mixture after the glycol titration was worked up in each case in the following manner. After dilution with water, the mixture was extracted repeatedly with ether. The ether was washed several times with water and evaporated to dryness. The residue was heated at 100° for 1 hour with a small volume of ethanolic potassium hydroxide in order to hydrolyze any acetylated product that might have been formed during the prolonged reaction in glacial acetic acid solution. On dilution of the saponification mixture with water a white solid was precipitated which was filtered off, washed with water, and dried.

The combined products from the first and second experiments, weighing 22.4 mg., were recrystallized three times from 50 per cent ethanol. 4.1 mg. of white crystals with a melting point of 233–234° were obtained. The material gave a negative Zimmermann ketone test.

2.588 mg. gave 7.480 mg. CO₂ and 2.610 mg. H₂O

C₂₁H₃₆O₃. Calculated. C 78.69, H 11.33

Found. " 78.85, " 11.29

When the sample was mixed with authentic pregnanediol (m.p. 236–237°), the melting point was 229.5–230.5°; mixed with authentic allopregnanediol (m.p. 238–240°), the melting point was 214–233°.

The crystalline forms of the unknown substance were examined from acetone, aqueous ethanol, and dioxane. The crystalline form was in each instance indistinguishable from that of pregnanediol crystallized from the same solvent.

From the products of the third and fourth experiments, an additional amount of 7.1 mg. of this substance was obtained. This was acetylated in the usual manner. The crude acetate, after sublimation at 135° and 0.04 mm. and crystallization from

methanol, melted at 164–165°.¹ The melting point was not depressed by admixture with authentic pregnanediol acetate. The crystalline form of the acetate of the unknown substance was indistinguishable from that of authentic pregnanediol acetate. The identification of the substance as pregnanediol was therefore considered to be complete.

The mother liquors from the crystallization of the pregnanediol, obtained from the first and second experiments, on evaporation yielded a gum which gave a strongly positive Zimmermann test. After treatment in the cold for 3 days with semicarbazide hydrochloride and sodium acetate in alcoholic solution, a white crystalline solid was obtained. After three crystallizations from ethanol this had a constant melting point of 264–265°.

2.288 mg. gave 5.870 mg. CO₂ and 1.990 mg. H₂O

C₂₃H₃₃O₃N₃. Calculated. C 69.10, H 9.58

Found. " 69.98, " 9.73

From the third and fourth lead tetraacetate oxidations was obtained in a similar manner a further quantity of this semicarbazone, which after two crystallizations melted at 262° with slight preliminary sintering. This melting point was unchanged after admixture with once crystallized semicarbazone of authentic 3-epihydroxyetiocholan-17-one which melted at 262° with slight preliminary sintering. The ketonic oxidation product was therefore identical with 3-epihydroxyetiocholan-17-one.

SUMMARY

The urines from two young women showing the adreno-genital syndrome have been shown to contain a new triol, m.p. 243–244°, of the formula C₂₃H₃₃O₃. This triol yields a diacetate, m.p. 136.5°. Oxidation with lead tetraacetate revealed the presence of one glycol group per molecule. The products of this oxidation were identified as acetaldehyde and 3-epihydroxyetiocholan-17-one. The triol is therefore pregnane-3, 17, 20-triol.

¹ The melting point of highly purified pregnanediol acetate is about 180°. Less carefully purified preparations usually show a double melting point, collapsing at about 160–165° and melting completely at about 180°. Sometimes the melt is complete at the lower temperature range.

The crude triol was found to be contaminated with appreciable amounts of pregnanediol.

The authors wish to express their warmest appreciation of the enthusiastic cooperation of Mr. L. R. Broster, of the Charing Cross Hospital, London, in this work. They are also deeply grateful to Dr. Jocelyn Patterson of the Charing Cross Hospital for the preparation of the urine concentrates. Grateful acknowledgment is made of gifts of androsterone and isoandrosterone from Professor L. Ruzicka of Zurich and the Ciba Company, Ltd., of Montreal, and of allopregnanediol from Dr. Desmond Beall.

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NITROGENOUS EXTRACTIVES OF SCALLOP MUSCLE

I. THE ISOLATION AND A STUDY OF THE STRUCTURE OF OCTOPINE*

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(Received for publication, May 10, 1937)

Of the guanidine derivatives occurring among the extractives of invertebrate muscle, arginine has been found most frequently (11). However, other guanidine compounds have been isolated. Iseki (9) reported that he obtained methyl agmatine from octopus muscle. Morizawa (18), also working with *Octopus octopodia*, obtained no arginine but guanidine and a base which he called octopine. Henze (5) may have previously isolated the latter compound from octopus. Only guanidine and agmatine were found in extracts of the sponge *Geodia gygas* (2).

Recently two guanidine derivatives, of particular interest because of their unexpected constitutions, have been isolated. Kutscher, Ackermann, and collaborators (12-14) have obtained from the muscle of *Arca noæ* arcaine, which they have identified as tetramethylene diguanidine, and Ackermann (1) has prepared from the starfish asterubine, which is dimethylguanidotaurine.

In the course of the present study, the arginine fraction of the aqueous extract of *Pecten* muscle yielded a crystalline base which was not arginine. The properties of the compound were identical with most of those described for octopine by Morizawa (18).

* This paper is taken from the thesis presented by Elinor Moore to the Faculty of the Graduate School of the University of Pennsylvania in 1936 in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Preliminary reports of this work have been presented before the American Society of Biological Chemists at New York, March 29-31, 1934, and at Washington, March 25-28, 1936, and before the Physiological Society of Philadelphia, May 20, 1935 (17).

However, Morizawa reports that octopine forms a strongly alkaline solution in water ("Die Lösung der freien Base reagiert gegen Lakmus stark alkalisch . . ."). He states that it was very difficult to obtain the CO_2 -free base in crystalline condition and does not report analyses of this product. Our compound yields in water a solution which is neutral to litmus and we had no difficulty in preparing the isoelectric material, free from combined CO_2 . Because of these differences between the descriptions of our compound and octopine we thought that the two compounds must be different and therefore called our material "pectenine" ((17) 1936). We were not successful in obtaining octopine from the Kyoto laboratory to compare with our material. However, in a recent paper (16) from the same laboratory, appearing after the completion of the present work, Mayeda reports the isolation of octopine from *Pecten yessoensis*, Jay.

In describing this octopine preparation Mayeda states that octopine picrate gives an intense blue color with Congo red paper, in contrast to arginine picrate (presumably the monopicrate) which gives an alkaline reaction to this indicator. As octopine picrate is a monopicrate and is acid to Congo red, it is to be assumed that the statements of Morizawa mentioned above regarding the basicity of octopine are incorrect. We find that the picrate of our compound which gives the same analytical data as Mayeda's octopine picrate forms in water a solution which is acid to Congo red paper. It would appear, therefore, that octopine and "pectenine" are identical and the name pectenine should be dropped.

Morizawa prepared the picrate and picrolonate of octopine and reported their analyses and some of their properties. He did not recognize that octopine was a guanidine derivative. We confirm all of Morizawa's findings except those mentioned above and in addition give data concerning the structure of the compound.

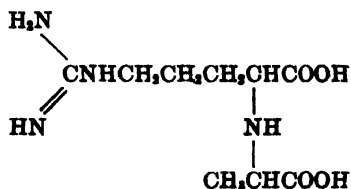
The free base which we have isolated crystallizes from dilute alcohol as colorless anhydrous needles, often in radiating clusters. It decomposes at $261\text{--}264^\circ$ (corrected) and shows an $[\alpha]_D$ of $+20^\circ$. It forms a crystalline picrate decomposing at $226\text{--}230^\circ$ (corrected) and a picrolonate decomposing at $237\text{--}239^\circ$ (corrected).

The analyses and molecular weight determination of octopine agree with the values calculated for the formula $\text{C}_9\text{H}_{13}\text{N}_4\text{O}_4$. The

high hydrogen percentage and negative tests for rings suggest a straight chain compound. Octopine was shown to contain a guanidine group by the Sakaguchi and acetylbenzoyl reactions and a secondary amine group by Liebermann's test. (Our preliminary note ((17) 1936) stated that the latter reaction was negative but this was subsequently found to be incorrect.) The compound contains no free amino nitrogen before or after boiling with HCl and no methoxy or N-methyl groups. The saturated (5 per cent) aqueous solution is neutral to litmus. In 90 per cent alcohol only one group (assuming the molecular weight to be 246) was titrated with NaOH and none with HCl.

Hydrolysis of octopine with barium hydroxide forms urea and an alcohol-insoluble compound decomposing at 256–257° (corrected). Analyses show the latter to be $C_8H_{16}N_2O_4$ and to contain one free amino group. These results establish the presence of a monosubstituted guanidine group in octopine. Upon oxidation with silver oxide the compound $C_8H_{16}N_2O_4$ yields 3 moles of CO_2 and 0.8 mole of NH_3 . Acetic acid and acetaldehyde have been identified among the products. Under similar treatment octopine itself gives 3 moles of CO_2 and 0.8 mole of NH_3 and acetaldehyde.

On the basis of the information mentioned above and on the supposition that the compound, coming as it does from invertebrate muscle, might be related to arginine, it is suggested that it may be a derivative of arginine formed by the substitution on the α -amino group with propionic acid attached through its α -carbon atom.¹



EXPERIMENTAL

The adductor muscles of the large sea scallop, *Pecten magellanicus*, were obtained in the market from a shipment kept on ice after having been dredged 4 or 5 days previously. Two prelimi-

¹ This structure has recently been proved to be correct by Irvin and Wilson (8).

nary analyses of trichloroacetic acid extracts of muscle had shown the presence of little or no arginine. The determinations were repeated on a solution made by extracting 100 gm. of the ground muscle with 150 cc. of 10 per cent trichloroacetic acid. The extract after having been nearly neutralized with sodium hydroxide and concentrated *in vacuo* at 50°, was diluted to a volume of 100 cc. It was analyzed for guanidine compounds by the arginase-urease and Sakaguchi methods. For the former the method of Hunter and Dauphinee (7) was adopted with all of the controls suggested. The quantitative adaptation of the Sakaguchi reaction was that of Weber (21) as modified by Jorpes and Thorén (10) except that a No. 62 Wratten green filter was used in the colorimeter. The suggested precautions were taken to insure the development of maximum color. The color given by the solution with the Sakaguchi reagents had a purple cast as described below for octopine and was quite different from that given by arginine. The arginase determination showed 0.013 gm. of arginine and the Sakaguchi determination 1.08 gm. of guanidine derivatives (calculated as arginine) per 100 gm. of muscle. From these results it is evident that, although scallop muscle from the market contains considerable amounts of material responding to the Sakaguchi reaction for guanidine compounds, only a small proportion of this can be accounted for by arginine.

In order to study the extractives of the muscle by isolation methods 6 kilos were ground in a meat chopper and extracted three times at 70–80° with a total of 8 volumes of water. Following filtration through muslin, the extracts were concentrated over a free flame with a fan to about two-thirds the volume of the muscle. The resulting concentrate was freed from glycogen and traces of protein by the addition of 5 volumes of 95 per cent alcohol.

The alcoholic filtrate was precipitated with mercuric sulfate in sulfuric acid and additional alcohol. After the solution had stood overnight in the ice box, the clear supernatant fluid was decanted and the precipitate removed and washed by centrifugation. The mercury precipitate was decomposed with hydrogen sulfide, the pH of the solution being maintained at about 3.0 by the addition of barium carbonate.

The filtrate from the mercuric sulfide, after concentration at 50° under reduced pressure, was fractioned with silver and barium

hydroxide by the Kossel-Kutscher technique. After removal of the purine and histidine fractions, the arginine fraction was precipitated at pH 7.2 to 11.0. This precipitate was suspended in water, acidified, and decomposed with hydrogen sulfide. Barium carbonate was added, if necessary, to prevent excessive acidity. When decomposition was complete (in 10 to 14 hours), the solution was aerated and the silver sulfide removed.

When silver nitrate had been used, the arginine fraction was reprecipitated with mercuric sulfate and alcohol and the precipitate treated as described below. The final solution of the arginine fraction was quantitatively freed of sulfate with barium hydroxide and decolorized with charcoal. After having been concentrated to a small volume under reduced pressure at 40–50° the filtrate was left in the refrigerator for several days for crystallization. After removal of these crystals a second crop was obtained by treating the mother liquor with alcohol. The yield of crude product from 6.1 kilos of tissue was 9.1 gm. in the first crop and 10.4 gm. in the second. The product was recrystallized by dissolving in the minimum amount of hot water, chilling, and adding alcohol until the appearance of turbidity. After crystallization had begun, the alcohol concentration was slowly increased to about 80 per cent.

The free base crystallized from dilute alcohol to form colorless anhydrous needles often in radiating clusters. In cold water it formed an approximately 5 per cent solution which was neutral to litmus. It was more soluble in hot water and insoluble in alcohol, ether, and acetone. The free compound melted with decomposition at 261–264° (corrected). With a 3.79 per cent aqueous solution, $[\alpha]_D^{24} = +19.6^\circ$. With a 2.41 per cent solution, $[\alpha]_D^{28} = +20.4^\circ$. Approximately the same value was obtained in 0.16 N HCl.

On slow heating the free base melted, then decomposed with charring and emission of the odor of burning hair. It left no residue. Elementary analysis by the sodium fusion method showed the absence of halogens, sulfur, and phosphorus and the presence of nitrogen. Absence of sulfur and phosphorus was corroborated after oxidation with sodium carbonate-potassium nitrate mixture.

$C_9H_{18}N_4O_4$. Calculated. C 43.92, H 7.37, N 22.76

Found. " 43.33, " 7.28, " 22.09 (micro-Kjeldahl)

The molecular weight of octopine was determined by the freezing point method. $C_9H_{18}N_4O_4$, calculated, 246; found, 240.

The compound was precipitated by phosphotungstic acid, silver salts with barium hydroxide or ammonia (soluble in excess NH_3), and mercuric sulfate with alcohol but not by alcoholic mercuric chloride except in the presence of sodium acetate. It was not precipitated from aqueous solution by mercuric sulfate, potassium bismuth iodide, potassium cadmium iodide, or potassium mercuric iodide. A sulfate, chloride, flavianate, gold chloride, or copper nitrate salt could not be obtained in crystalline form.



FIG. 1. Octopine picrate crystals

With picric acid, octopine formed yellow anhydrous needles (Fig. 1) melting with decomposition sharply at $226-230^\circ$ (corrected), depending upon the rate of heating. The picrate could be dissolved in cold water to form a 0.3 per cent solution. Since it was about 10 times more soluble in hot water, it could be recrystallized readily.

$C_9H_{18}N_4O_4 \cdot C_6H_3N_3O_7$.	Calculated.	C 37.88, H 4.45, N 20.63
	Found.	" 38.08, " 4.51, " 20.18 (Kjeldahl)

Picric acid was determined by the gravimetric method of Cope and Barab (3). Calculated, picric acid 48.2 per cent; found 48.2 per cent.

The picrolonate crystallized as burrs of yellow microscopic needles which upon rapid heating decomposed at 237–239° (corrected).

$C_8H_{11}N_3O_4$, $C_{10}H_{13}N_3O_4$. Calculated, N 21.96; found, N 21.40

Octopine decolorized a dilute $KMnO_4$ solution somewhat more slowly than did arginine. It gave a positive pine splinter test. In solution it dissolved copper carbonate to yield a deep blue color. The Sakaguchi and acetylbenzoyl reactions for guanidine compounds were positive, but arginase liberated no urea. Negative results were obtained with the Jaffe test, before and after boiling with HCl , and with the Millon, Wurster, ninhydrin, biuret, xanthoproteic, Hopkins-Cole, and diazo reactions. The Liebermann test for secondary amines was positive. The compound showed no free amino nitrogen by the Van Slyke method before or after boiling with 4 N HCl for 2 hours and no methoxy or N -methyl groups.

The presence of titratable acidic and basic groups was investigated by the technique described by Harris (4). Samples of a solution of the base to which enough boiled water had been added to make a total volume of 5 cc. were treated with 50 cc. of alcohol which had been redistilled from calcium oxide. A control was titrated with sodium hydroxide to the first appearance of blue with thymolphthalein (pH 9.3 to 10.5) and the other solutions were titrated to match it. The same solutions were then similarly titrated with hydrochloric acid to the end-point of methyl red (pH 5.2 to 5.6). Calculations were made on the basis of a molecular weight of 246. The fraction of 1 mole titrated with $NaOH$ was 0.98 and that with HCl was 0.0.

Quantitative comparisons of the intensity of color given by arginine and octopine in the Sakaguchi reaction (10, 21) were made with arginine as the standard. The color given by the base is different from that given by arginine or any of the several other guanidine compounds tested. It is a purplish red (20), as contrasted with the more orange-red produced by arginine. Therefore a No. 62 Wratten filter (maximum transmission at λ 530) was used in making the comparisons. It was found that octopine (assuming a molecular weight of 246) gave 20 per cent greater absorption of light in this region than an equimolecular quantity of arginine.

In the acetylbenzoyl reaction as developed by Lang (15) ar-

ginine and octopine yielded the same color, but again the latter gave a greater intensity than an amount of arginine containing an equivalent quantity of the guanidine group.

In order to gain further information concerning the structure of octopine, two types of procedure were used; first, hydrolysis with alkali (Schulze and Winterstein (19)) and, second, oxidation with silver oxide (Herbst and Clarke (6)).

Hydrolysis by Barium Hydroxide—1.2 gm. of octopine were gently refluxed for 1 hour with 3.3 gm. (2.15 moles) of barium hydroxide in 100 cc. of water (19). After removal of the barium as barium sulfate, the solution was concentrated to a thick syrup under reduced pressure. Analysis by the urease method of the fraction extracted with hot alcohol showed 52 per cent of the calculated amount of urea. From this fraction crystals typical of urea nitrate were obtained. These were converted into the xanthidrol derivative, which after recrystallization from pyridine decomposed at 261–262° (corrected). A sample of known dixanthidryl urea and a mixture of the two decomposed at 261–262°. $C_{27}H_{30}N_2O_8$, calculated, N 6.67; found, N 6.57.

The residue from the alcohol extraction was freed from alcohol, dissolved in water, and analyzed for amino nitrogen by the Van Slyke method, which yielded 61 per cent of the expected value. The solution gave positive Wurster and negative ninhydrin tests. The color by the Sakaguchi reaction was roughly equivalent to 0.5 gm. of octopine. The solution upon fractional crystallization yielded two crops of crystals. The more soluble, 0.42 gm., was shown by a Sakaguchi color value and isolation of the picrate to consist mainly of the original base. The less soluble fraction, after two recrystallizations from water by the addition of alcohol, weighed 0.44 gm. A 1.77 per cent solution read in a 1 dm. tube gave $[\alpha]_D^{21} = +13.6^\circ$. Several more recrystallizations yielded a product decomposing at 256–257° (corrected). The compound formed colorless needles having no water of crystallization. It contained no sulfur or halogens. The Sakaguchi reaction was negative.



Calculated. C 47.03, H 7.90, N 13.72, NH_2 -N 6.86

Found. " 46.61, " 7.89, " 13.09, " 5.98 (3 min.)
" 7.23 (10–30 min.)

Silver Oxide Oxidation of the Compound Produced by Barium Hydroxide Hydrolysis of Octopine—The procedure of Herbst and Clarke (6) for the oxidation was adopted with very few modifications. A suspension of moist nitrate-free silver oxide from 12 moles of silver nitrate was brought to a boil on a hot-plate and 1 gm. of the hydrolytic product suspended in 35 cc. of warm water added. A receiving train consisting of a flask charged with HCl and two centrifuge bottles containing filtered saturated barium hydroxide solution was immediately attached. At the end of 7 hours of gentle boiling the rate of evolution of carbon dioxide and

TABLE I
Determinations of Products Formed by Action of Silver Oxide on Hydrolytic Product

Determination	Calculated result	Yield	Per cent yield
Experiment 1			
Silver, moles	4-8	9.96	249-125
Ammonia, mole	1	0.80	80
Other volatile bases, moles		0.11	
NH ₃ -N in 3 min., mg.	68.6	28	41
Total N, mg.	68.6	50	73
Volatile acids by titration, cc. 0.1 N	49	34.6	71
Crude acetaldehyde, calculated, mg.	216	53	25
Experiment 2			
CO ₂ by titration, moles	2	2.97	148
Volatile bases, mole	1	0.94	94
NH ₃ -N in 3 min., mg.	110	41	38

ammonia had become so small that the reaction was stopped. The receiving train was disconnected and the first receiver boiled to drive all the carbon dioxide into the barium hydroxide. A pyrrolidine-like odor was noticed.

The barium carbonate, after having been washed with boiled water, was dissolved in excess standard hydrochloric acid. The barium was estimated by titration of the excess acid and by gravimetric determination as barium sulfate (Table I, Experiment 2). The contents of the first receiver were distilled while acid. The distillate had a strong odor of acetaldehyde and con-

tained no titratable acids. It gave positive Tollen's and fuchsin tests but a negative resorcinol test for formaldehyde. The aldehyde was precipitated as the dimethyldihydroresorcinol derivative, giving a yield of crude material amounting to 0.37 gm. After several recrystallizations the compound melted at 139° (uncorrected). The anhydride prepared from the dimethyldihydroresorcinol derivative melted at 175° (uncorrected). Both melting points were the same as those of the corresponding known acetaldehyde derivatives and of the mixtures of the known with the material isolated. The residue from the acetaldehyde distillation, after having been made alkaline with sodium hydroxide, was

TABLE II

Determinations of Products Formed by Action of Silver Oxide on Octopine

Determination	Calculated result	Yield	Per cent yield
Silver, moles	4-12	17 1	428-143
CO ₂ by titration, moles	2	2 94	147
Ammonia, mole	1	0 83	83
Other volatile amines, mole		0 02	
NH ₃ -N in 3 min., mg.	None	16 5	
Sakaguchi as γ -guanidinebutyric acid, mg.	1179	274	23
Crude acetaldehyde, calculated, mg.	358	125	35

distilled into a measured amount of standard acid. After the excess acid had been titrated, the volatile bases were differentially determined by the method of Weber and Wilson ((22) Table I).

The reaction mixture was filtered through a Jena crucible to remove the silver residue which was freed of salts and of oxide by repeated washing with hot dilute acetic acid and ammonia. After dissolving in nitric acid, the metallic silver was determined gravimetrically as silver chloride (Table I). The filtrate from the silver residues was acidified and treated with H₂S to remove the silver. Amino nitrogen was determined by the Van Slyke method and total nitrogen by the micro-Kjeldahl technique (Table I). This solution gave no test for aldehyde with Schiff's reagent. Since, after all mineral acid had been removed, the solution was markedly acid to litmus, it was treated with sulfuric acid and repeatedly distilled under reduced pressure to a small volume. The distilla-

tion was repeated until only very small amounts of titratable acid were present in the distillate (Table I). From the combined distillate 96 mg. of silver acetate were obtained.

$C_7H_{11}O_2Ag$. Calculated, Ag 64.64; found, Ag 65.25

A second experiment (see the last part of Table I) yielded similar results.

Silver Oxide Oxidation of Octopine—2 gm. of the octopine were oxidized with 18 equivalents of silver oxide in 250 cc. of water. The reaction was stopped at the end of 13½ hours. Quantitative studies of the products were conducted as before (Table II). The value for silver is probably too high because of the great difficulty in washing so bulky a residue. Breakdown of succinic acid, which was expected but not found, might also account for the high silver value and for the high yield of carbon dioxide. Acetaldehyde was again identified, 0.87 gm. of the crude dimethyldihydroresorcinol derivative being obtained. The reaction solution was analyzed by the Van Slyke amino nitrogen and Sakaguchi methods. In the latter reaction the color obtained was that of arginine rather than that of octopine. No product from this fraction was identified.

SUMMARY

The isolation of octopine from the adductor muscle of *Pecten magellanicus* is described. Some of the properties were studied.

Experimental data are given which suggest that the compound is a derivative of arginine formed by the substitution on the α -amino group with propionic acid attached through its α -carbon atom.

The authors wish to express their gratitude to Dr. H. T. Clarke of Columbia University for his suggestions concerning the determination of the structure and to Dr. H. B. Vickery of the Connecticut Agricultural Experiment Station for generously supplying samples of some of his preparations.

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NITROGENOUS EXTRACTIVES OF SCALLOP MUSCLE

II. ISOLATIONS FROM AND QUANTITATIVE ANALYSES OF MUSCLES FROM FRESHLY KILLED SCALLOPS*

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The failure to isolate arginine from the adductor muscles of *Pecten magellanicus* (purchased in the market), as described in Paper I, preceding, was unexpected in view of the fact that arginine or phosphoarginine had been reported in various mollusks and other invertebrates including different species of *Pecten* (5, 8, 9). Our demonstration that another guanidine compound, namely octopine, was present in *Pecten magellanicus* (11) led to the thought that this compound might be the hydrolysis product of a phosphagen different from phosphoarginine. Other phosphagens have been thought to occur in invertebrates (1, 12) although none has been isolated (6).

In order to study the phosphagen of *Pecten magellanicus* we carried through a procedure for isolation upon extracts of the adductor muscles obtained from living animals. Phosphoarginine was isolated in a yield representing only a small percentage of the easily split phosphate in the extract. No other phosphagen was obtained. These extracts also yielded both arginine and octopine, the presence of both of which compounds was confirmed by analyses with the arginase and Sakaguchi methods.

* This paper is taken from the thesis presented by Elinor Moore to the Faculty of the Graduate School of the University of Pennsylvania in 1936 in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

A preliminary report of this work was presented before the American Society of Biological Chemists at Washington, March 26-28, 1936 (*Proc. Am. Soc. Biol. Chem.*, 8, lxxi (1936); *J. Biol. Chem.*, 114 (1936)).

EXPERIMENTAL

Isolation of the Phosphagen—The isolation procedure was based upon those of Meyerhof and Lohmann (10) and of Fiske and Subbarow (2). *Pecten magellanicus*, living but in poor condition, served as the starting material. With the usual precautions to retard hydrolysis of the labile phosphates, a trichloroacetic acid extract was made of the entire adductor muscles (7). After precipitation of the glycogen with 2 volumes of alcohol, the filtrate was saturated with powdered barium hydroxide. The resulting precipitate, after centrifugation, was extracted once with an equal volume of water with mechanical stirring for 1 hour and twice with 0.5 volume for 30 minutes. From these extracts the phosphagen salt was separated by the slow addition of 2 volumes of absolute alcohol. The precipitate was dissolved in the minimum amount of water and after filtration of the solution was reprecipitated with absolute alcohol.

The barium was then removed with a slight excess of sulfuric acid and the free phosphagen was thrown out with 8 to 10 volumes of absolute alcohol. The phosphagen was transformed to the calcium salt by dissolving in water and treating with a suspension of fresh calcium hydroxide until the solution was alkaline to litmus. The solution was then made slightly acid to litmus with hydrochloric acid and treated with 2 volumes of absolute alcohol. The product was reprecipitated until there was no further improvement in the purity. The precipitate was not crystalline. The percentages of total N, amino N, and P in the preparation were about 15 per cent below the theoretical values for the primary salt of phosphoarginine. The calcium was about 10 per cent high.

Arginase-urease (3) and Sakaguchi (4) determinations were made before and after hydrolysis with 0.1 N acid. Prior to hydrolysis the material showed by the former method 5.5 per cent of free arginine and by the latter an extremely pale color. The analyses of the hydrolyzed material gave the following results (corrected for water content).

(C ₆ H ₁₄ N ₄ O ₆ P) ₂ Ca. Calculated.		Arginine 63.74	
Found, arginase method.		"	50.8
" Sakaguchi "		"	50.7

The value of the hydrolysis constant of the product isolated was 6.7×10^{-3} for about 60 per cent decomposition at pH 1.2 and 28°.

The velocity of hydrolysis was slightly greater at pH 2 than at pH 1. The molybdate inhibition (0.25 per cent ammonium molybdate) was 20 to 45 times. Evidently the compound was impure phosphoarginine, possibly contaminated with calcium carbonate.

Guanidine Derivative Content of Extracts of Fresh Muscle—In view of the isolation of phosphoarginine from fresh muscle and the finding of octopine but no arginine in market muscle it was thought desirable to determine whether arginine and octopine were present in the fresh tissue extracts. For this purpose a composite solution consisting of samples from the trichloroacetic acid extracts of three phosphagen preparations was used. After the solution had been nearly neutralized, concentrated under reduced pressure at 50–55°, and made up to a convenient volume, it was hydrolyzed and analyzed by the arginase-urease and Sakaguchi methods. The arginine found by the arginase method indicated that 770 mg. of arginine were present in 100 gm. of muscle; the Sakaguchi method gave a color equivalent to 1100 mg. of arginine per 100 gm. of muscle. Assuming that the excess color by the Sakaguchi method was due to octopine, the corrected results show that about 75 per cent of the total guanidine (Sakaguchi) was present as arginine in the extract of fresh tissue. This is in striking contrast with market muscle, which contained little or no arginine.

Isolation of Arginine and Octopine—A portion of the combined alcoholic filtrates from two phosphagen preparations, which had been preserved slightly acidified in the refrigerator for several months, was used for the isolation. The sample represented 1.46 kilos of muscle. After the barium had been removed with sulfuric acid, a mercury-alcohol precipitation and a Kossel-Kutscher silver fractionation were carried out as described in Paper I.

The concentrated solution of the decomposed silver-arginine fraction was treated with the calculated amount of flavianic acid. Characteristic gleaming orange plates of arginine flavianate separated copiously. After decomposition of the flavianate 2.81 gm. of arginine monohydrochloride were obtained. The product melted with decomposition at 225° (corrected), while a known sample melted with decomposition at 222° and a mixture at 224°. It yielded 99 per cent of the expected amount of ammonia by the arginase-urease method.

$C_6H_{14}N_4O_2HCl$.	Calculated.	N 26.50,	NH_2-N 6.65
	Found.	" 26.49,	" 6.78

The filtrate from the arginine flavianate after concentration under reduced pressure at 45–50° was freed of flavianic acid by extraction with *n*-butyl alcohol. Quantitative removal of sulfate with barium hydroxide left a neutral solution in contrast to the alkaline solution observed before removal of the arginine. Addition of picric acid to the concentrated filtrate from the barium sulfate yielded 5.56 gm. of crystals. The final yield after many recrystallizations was 2.46 gm. The product melted with decomposition at 228° (corrected). Simultaneously a mixture with octopine picrate melted with decomposition at 228.6°. No amino nitrogen was found by the Van Slyke method.

$C_8H_{12}N_4O_4 \cdot C_8H_8N_2O_7$. Calculated. C 37.88, H 4.45, N 20.63
Found. " 37.98, " 4.40, " 19.82

The properties and composition indicate that the isolated material was octopine picrate.

SUMMARY

1. Phosphoarginine has been identified in the extracts of the fresh adductor of *Pecten magellanicus*.
2. The presence of arginine along with another guanidine compound has been demonstrated quantitatively.
3. Arginine and octopine have been isolated from extracts of fresh tissue.

The authors are very grateful to Dr. H. B. Baker of the Department of Zoology for the identification of the animals.

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RATE OF EVAPORATION IN SERUM AS A MEASURE OF VAPOR PRESSURE, OSMOTIC PRESSURE, AND CONCENTRATION OF SOLUTES*

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In a previous communication (2) a method devised by Hill (3) for the measurement of vapor pressure of whole blood was described. The apparatus consists of a symmetrically wound thermopile so constructed that the solution under examination is permitted to evaporate from one face of the instrument while a standard solution (0.92 per cent of sodium chloride) simultaneously evaporates from the opposite face, the whole system being at constant temperature. The difference in rate of evaporation of the solutions produces an E.M.F. which is recorded on a galvanometer; thus, the unknown sample may be characterized in terms of the concentration of a solution of sodium chloride which possesses an identical rate of evaporation.

The application of this method to the measurement of vapor pressure in serum and in whole blood has met with some criticism, inasmuch as the pressure is not measured directly. What is measured is the rate of evaporation of the sample. Although with simple solutions of electrolytes strict proportionality exists between vapor pressure and rate of evaporation, it has been argued that in serum the formation of a film on the surface may retard the loss of moisture and that with whole blood the red cells may significantly alter the surface available for evaporation.

Modification of the rate of evaporation by such interfering phenomena might be expected to produce wide and unpredictable divergence between the results of duplicate determinations. It is

* A preliminary report (1) of this investigation was made at the meeting of the Society for Pediatric Research at Atlantic City, May 5, 1936.

therefore important to note that the measurements are reproducible within a probable error of less than 0.5 per cent, a fact which at least indicates that some stable property of the blood is being measured. Reproducibility of results, while supporting, does not necessarily prove the contention that in serum or in whole blood the rate of evaporation is a measure of the vapor pressure. Because vapor pressure can be used for calculating osmotic pressure and because there is need of a method for estimating osmotic pressure in biological fluids which does not involve the extreme environmental conditions of freezing point determinations, it appeared worth while to secure additional data in support of the validity of determining vapor pressure by the thermoelectric method. These data were obtained with serum; the findings with whole blood which have been reported previously still require critical examination.

In previous papers in which measurements made by the thermoelectric method were recorded (2, 4) and in which the validity of the method was not being examined, the results were regarded and spoken of as measures of vapor pressure or of osmotic pressure. For clarity in the ensuing discussion it is necessary to distinguish between vapor pressure and that measure of the rate of evaporation of a sample of serum which is given by the thermoelectric procedure; the term "equivalent vapor pressure" will be used to refer to the latter. The standard of reference in the thermoelectric method is a solution of sodium chloride of known concentration. When an unknown solution is measured against the standard of reference, the resulting deflection of the galvanometer results from and is proportional to the difference between the rates at which the two solutions are evaporating. Equivalent vapor pressure is therefore conveniently expressed in terms of that concentration of a solution of sodium chloride which if it had been placed opposite to the standard would have produced an identical deflection of the galvanometer. If it can be shown that the equivalent vapor pressure of serum is a function of the same property which determines the true vapor pressure and the osmotic pressure, it will then be clear that there is a direct linear relationship between any two of the three properties: equivalent vapor pressure, osmotic pressure, and lowering of true vapor pressure from that of water; the relationship between these

three properties and the absolute value of vapor pressure expressed in pressure units is inverse.

Proof of the contention that the rate of evaporation of serum is a measure either of vapor pressure or of osmotic pressure would seem to be furnished if it were possible to determine quantitatively all of the separate constituents of serum, to calculate for each constituent its equivalent osmotic concentration, and finally to demonstrate that the sum of the calculated concentrations was equal to the observed equivalent vapor pressure of the serum. Expressed as an equation the calculation would take the form

$$\varphi_1[\text{Na}] + \varphi_2[\text{Mg}] + \varphi_3[\text{Ca}] + \cdots \varphi_n[m] = [V]$$

where the various constituents are expressed in molecular or ionic concentrations, where the φ values represent osmotic activity coefficients, and where V is the equivalent vapor pressure. From the thermodynamic standpoint such a demonstration is impossible; moreover in this simple form it obviously could not express the relationship accurately, since it neglects the possibility of chemical combination between two or more constituents. An example of such a combination is the effect of protein on the ionization of calcium. In the present state of knowledge and with the limitations imposed by available analytic procedures, it is impossible to allow for all similar effects in a way which would inspire confidence in the result.

In view of the difficulty of effecting a comprehensive demonstration such as that described, evidence for the significance of measurements of equivalent vapor pressure was sought in another way. In a group of sera the equivalent vapor pressure was measured and a selected number of the osmotically active constituents were determined by chemical analysis; a relationship was then looked for between the results of all analyses and the equivalent vapor pressure. Instead, however, of assuming a theoretical osmotic effect for any constituent, the data were examined mathematically by the method of multiple correlation to determine the existence of any quantitative relationship between equivalent vapor pressure and chemical composition. This method of examination is based on the theory of least squares; it leads to the formulation of regression equations by means of which the equivalent vapor pressure can be predicted from the constituents

of serum in the best¹ way that the data will permit. It was felt that such a study would support the assumption that equivalent vapor pressure is functionally related to osmotic pressure and vapor pressure, if two goals were realized: (1) that the agreement between predicted and determined values of equivalent vapor pressure was reasonably close; (2) that the regression equation assumed a form consistent with accepted concepts of the nature of osmotic pressure. To an extent which will appear both of these objectives have been reached.

In a preliminary investigation, in addition to measurement of equivalent vapor pressure, the sera were analyzed for the following constituents: total fixed base, non-protein nitrogen, protein, bicarbonate, chloride, and in a number of instances sugar. The preliminary study of correlation demonstrated the feasibility of this method of approach; it also showed that the inclusion of so many variables would have a negligible effect in improving the accuracy of predictions of equivalent vapor pressure. Moreover, justification for omission of some of the items can be adduced on theoretical grounds. The final study, which included 53 samples of serum, was therefore limited to measurements of equivalent vapor pressure, total fixed base, non-protein nitrogen, and protein.

It has been stated that one purpose in treating these analytic data by the method of multiple correlation was to learn whether the final regression equation would have a form consistent with accepted concepts of the nature of osmotic pressure. It is easiest to visualize what this form should be by portraying the composition of serum diagrammatically (Fig. 1) in a manner similar to that originated by Gamble (5). In the usual Gamble diagram the concentrations of the various constituents of serum are expressed as milli-equivalents per liter; in Fig. 1 the concentrations are expressed on a molal basis, that is as milli-equivalents per 1000 gm.

¹ When regression equations with more than one independent variable are being computed, it is necessary to postulate linearity for all of the relationships. For most of the simple solutes of serum it is known that vapor pressure and osmotic pressure vary in a linear manner with concentration throughout the range encountered in physiologic solutions. The word "best," as we have used it, is valid only in so far as the assumption of linearity of regression is correct.

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of serum water. This distinction is of no importance when it is desired merely to represent the balance between negative and positive electrolytes; it is, however, desirable in the present case, since osmotic pressure and vapor pressure are functions of molal concentration. It will be shown later that a relationship which is useful from the practical rather than theoretical standpoint emerges even when the distinction is not made. It is generally believed that the fixed base of serum exists for the most part in osmotically active form; only a small portion of the total base, namely the undissociated fractions of calcium and magnesium, is devoid of appreciable effect. The total base (represented by

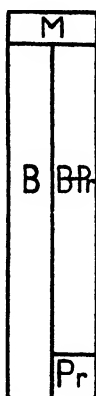


FIG. 1. Diagrammatic representation of the chemical composition of serum. *B* represents total base; *Pr*, osmotic effect of protein; *B - Pr*, osmotically active electrolyte; *M*, non-protein nitrogen.

B in Fig. 1) can be regarded as contributing positively to the osmotic pressure. On the anion side of the diagram the constituents of greatest importance are chloride (Cl^-) and bicarbonate (HCO_3^-); smaller contributions to the osmotic pressure are made by sulfate (SO_4^-), phosphate (HPO_4^- and H_2PO_4^-), and organic acids. Because of the large size of the molecule the osmotic effect of protein (*Pr*) is negligible. Since the sum of all the anions must equal the total base, the osmotically active anions are represented by *B - Pr*; it follows that the osmotically active electrolyte, both anions and cations, is represented by $2B - Pr$. The usual Gamble diagram does not portray the non-electrolyte

constituents of serum; it is obvious that such constituents contribute to the osmotic pressure but unlikely that they rank in importance with the electrolyte. To measure by quantitative analysis all or even many of the non-electrolytes was impracticable. The single constituent of this group which may appear in large amounts in serum and which, because of its small molecular size, exerts an appreciable osmotic pressure is urea. Measurements of urea might well have been used in this study to provide a rough appraisal of the contribution of non-electrolytes to osmotic pressure. Actually the total non-protein nitrogen was selected rather than its urea fraction, because the quantity of serum was often limited and non-protein nitrogen estimations were always obtained in the process of determining protein. The difference here is probably of minor importance. It is clear that the final regression equation should include non-protein nitrogen (M) as a positive contributor.

The relationship to be expected from the foregoing concept can be approximate only; the exact value of the coefficients in the regression equation cannot be predicted in advance. However, it can be seen that the equation should take the following form:

$$[V] = 2C_1B - C_2Pr + C_3M + C_4$$

Here C_1, C_2, C_3 are constants which determine the exact values for the several coefficients and C_4 is a constant which may be thought of as the average concentration of osmotically active constituents not otherwise represented in the equation. However, it is improbable that C_4 can have true physiologic significance, since it may be determined in part by mathematical considerations alone, such, for instance, as failure of linearity of regression in one of the independent variables. The important point to note, however, regarding the form of the equation is that the coefficients which introduce B and M are positive, whereas that which modifies Pr is negative.

Units of Measure—In the foregoing description the several constituents of serum are expressed in terms of molecular concentration; it is assumed that the equivalent vapor pressure is to be predicted in terms of molecular concentration. Such a mode of expression is obviously impossible in the case of non-

protein nitrogen. Moreover, since all of the variables in the regression equation will be modified by constants, it is clear that the use of any more convenient unit of measure for a constituent will result only in a change in the value of the respective constant. For this reason we have not been consistent in expressing all constituents in the same units; the forms adopted are those which have become familiar by common usage. The units of measure as well as the symbols by which the various constituents will be designated in the equations are as follows:

Units and Abbreviations

V = equivalent vapor pressure in gm. NaCl per 100 gm. H₂O

b = total fixed base in m.-eq. per liter serum

p = protein in gm. per 100 cc. serum

n = non-protein nitrogen in mg. per 100 cc. serum

B = total fixed base in m.-eq. per 1000 gm. serum water

P = protein in gm. per 100 gm. serum water

N = non-protein nitrogen in mg. per 100 gm. serum water

Water in 100 cc. serum = (99.0 - 0.75*p*) gm.

R = coefficient of multiple correlation

P.E. (est.) = probable error of estimate

Methods

Samples of Serum—Blood was collected under liquid petrolatum, allowed to clot, and the serum separated by centrifugation. After the serum had been transferred to another tube, no further attempt was made to prevent access of air. The majority of samples was obtained from healthy subjects; with the intention of securing wide variations in the various constituents to be studied, the remaining samples were taken from patients with various illnesses. Several infants with intestinal intoxication yielded sera low in non-protein nitrogen; high values were obtained in severe renal insufficiency; the lowest and highest non-protein nitrogen values in the series were, respectively, 12.5 and 236.0 mg. per 100 cc. With total base, variations from the normal were obtained in infants with intestinal intoxication and in children with lobar pneumonia; the lowest and highest levels were 128 and 162 milli-equivalents per liter. Variations in serum protein were provided by patients with chronic malnutrition and by samples of serum from the veins of the foot in healthy subjects who had remained standing for from 20 to 30 minutes; the range

for serum protein was from 3.32 to 8.30 gm. per cent. The resulting range for equivalent vapor pressure was from 0.7670 to 1.0859 gm. per cent of sodium chloride. Sera with pathologically high values for lipid or sugar were not included in the series.

Chemical Methods—The total fixed base of the serum was determined by Hald's (6) microgravimetric modification of the benzidine method. Of the solution of ash, aliquots were taken which were equivalent to either 0.4 cc. or 0.8 cc. of serum and which yielded precipitates of about 8 mg. or 16 mg., respectively. The lighter precipitates were weighed with a microanalytic balance. The following formula was used to convert the weight of precipitate into milli-equivalents of base per liter of serum: $(1000 \times \text{weight of precipitate equivalent to 1.0 cc. of serum})/141 = \text{milli-equivalents per liter}$ (7). Nitrogen was measured as ammonia after micro-Kjeldahl digestion. With total nitrogen and the higher levels of non-protein nitrogen the ammonia was distilled in steam into N/70 hydrochloric acid and titrated back with N/70 sodium hydroxide. With the lower values for non-protein nitrogen the ammonia was determined by nesslerization and comparison with a standard in a colorimeter. Protein was calculated as 6.25 times the difference between total nitrogen and non-protein nitrogen. All chemical analyses were performed in duplicate.

Mathematical Aspects—The formula for computing the water content of serum, which is given in the list of units, was taken from McLean and Hastings (8). The formulas for calculating partial and multiple coefficients of correlation, regression equations, and errors of estimate are given by Garrett (9). Correlations were made by the long method, that is without the use of scatter diagrams; through all computations an accuracy of six significant figures was maintained.

EXPERIMENTAL

Relationship in Serum between Equivalent Vapor Pressure and [Total Base, Non-Protein Nitrogen, and Protein]—The results of all 53 analyses are shown in Table I. When these data are correlated with V as the dependent variable and with B , N , and P as independent variables, the following relationships emerge:

$$\begin{aligned}
 R_{V(B, N, P)} &= 0.9630 \\
 V &= 0.008705B - 0.002978P + 0.001142N + 0.23745 \quad (1) \\
 \text{P.E. (est. } V) &= 0.01042
 \end{aligned}$$

The coefficient of multiple correlation, R , is so high as to leave little room for doubt that the relationship expressed by the regression equation is one of cause and effect. The probable error of estimate is 1.2 per cent of the mean value for determined equivalent vapor pressure. It may be concluded that the equivalent vapor pressure of serum gives a correct indication of the concentration of its solutes. The agreement between predicted and determined values of the equivalent vapor pressure is shown in Fig. 2; the figure gives a graphic representation both of the coefficient of multiple correlation and of the probable error of estimate. The coefficients in the regression equation which modify B and N are positive; the coefficient which modifies P is negative. This form of the equation supports the assumption that the equivalent vapor pressure of serum is functionally related to its true vapor pressure and its osmotic pressure.

Relationship in Serum between Total Base and [Equivalent Vapor Pressure, Non-Protein Nitrogen, and Protein]—In view of the close interrelationship among the variables, the suggestion at once presents itself of reversing the process and attempting to predict total base from measurements of equivalent vapor pressure, non-protein nitrogen, and protein. It need scarcely be mentioned that regression equations are not simple algebraic equations and that the desired formula for predicting base cannot be obtained by solving Equation 1 for B . However, it is readily secured by the same process which led to Equation 1 when total base is treated as the dependent variable and vapor pressure, non-protein nitrogen, and protein as independent variables. The following relationships emerge:

$$\begin{aligned}
 R_{B(V, N, P)} &= 0.8531 \\
 B &= 169.96V - 0.1936N + 1.481P + 11.65 \quad (2) \\
 b &= (0.99 - 0.0075p)B \quad (3) \\
 \text{P.E. (est. } b) &= 2.096
 \end{aligned}$$

Equation 2 is used for predicting base in terms of milli-equivalents per 1000 gm. of serum water; Equation 3 is used for expressing this result in the usual form of milli-equivalents per liter of

serum. The relationship between found and predicted values for total base is represented graphically in Fig. 3. In comparing $R_{B(V,N,P)}$ with $R_{V(B,N,P)}$ it should be noted that the lower value of

TABLE I

Equivalent Vapor Pressure and Chemical Constituents in 53 Sera

The data are in ascending order of the values for equivalent vapor pressure.

Sample No.	Equivalent vapor pressure	Fixed base	Non-protein N	Protein	Sample No.	Equivalent vapor pressure	Fixed base	Non-protein N	Protein
	gm. per cent NaCl	m.-eq. per l.	mg. per 100 cc.	gm. per 100 cc.		gm. per cent NaCl	m.-eq. per l.	mg. per 100 cc.	gm. per 100 cc.
1	0.7670	135.7	16.8	6.05	27	0.8468	151.6	30.4	7.47
2	0.7932	139.3	14.9	3.32	28	0.8469	151.8	37.6	9.10
3	0.7948	143.9	12.5	3.70	29	0.8474	151.7	35.2	7.46
4	0.8068	143.3	20.4	7.23	30	0.8479	150.7	26.4	7.60
5	0.8128	143.2	18.8	5.15	31	0.8488	151.3	27.6	6.94
6	0.8208	145.8	31.0	6.03	32	0.8503	149.7	28.4	6.94
7	0.8236	147.6	31.2	6.33	33	0.8512	151.7	31.6	8.17
8	0.8248	147.4	20.6	5.72	34	0.8539	155.8	19.7	5.24
9	0.8257	152.2	28.4	6.75	35	0.8540	155.5	24.8	6.97
10	0.8311	151.8	31.2	6.80	36	0.8554	151.8	30.4	6.57
11	0.8313	144.8	22.4	5.92	37	0.8575	151.8	36.4	7.19
12	0.8314	127.6	116.8	5.45	38	0.8578	153.2	27.2	6.80
13	0.8320	149.9	32.8	6.96	39	0.8594	151.5	30.4	6.99
14	0.8357	149.9	29.6	7.08	40	0.8594	153.5	32.8	7.65
15	0.8379	148.6	25.2	7.04	41	0.8604	155.7	30.0	7.25
16	0.8384	156.0	29.6	6.69	42	0.8620	149.3	28.3	7.54
17	0.8386	150.7	24.4	7.08	43	0.8630	151.2	48.0	8.53
18	0.8388	151.3	25.2	6.65	44	0.8790	152.7	37.8	6.74
19	0.8392	150.8	32.8	7.33	45	0.8838	151.9	25.2	7.06
20	0.8413	149.9	36.9	8.05	46	0.8910	157.7	28.1	6.80
21	0.8419	152.4	32.8	6.98	47	0.8942	155.2	34.9	8.30
22	0.8428	150.5	33.2	6.83	48	0.9112	155.7	52.3	5.44
23	0.8430	145.3	21.2	7.80	49	0.9612	146.6	159.2	5.20
24	0.8438	148.4	24.4	7.75	50	0.9695	144.9	108.8	8.06
25	0.8450	153.5	30.8	7.10	51	0.9715	149.3	138.4	4.67
26	0.8454	148.0	27.2	7.62	52	1.0735	161.9	182.4	5.33
					53	1.0859	146.3	236.0	5.98

the former arises chiefly from the fact that the range of observations was considerably less with *B* than with *V*. Still it can be shown that the error in predicting base is somewhat greater

than in predicting equivalent vapor pressure. In the case of base the probable error of estimate is 1.40 per cent of the mean base value, whereas with equivalent vapor pressure the probable error is 1.21 per cent of the mean value.

Relationship in Serum between Equivalent Vapor Pressure and [Total Base and Non-Protein Nitrogen]—In order to simplify the procedure of obtaining estimates of equivalent vapor pressure a regression equation was calculated in which the least important

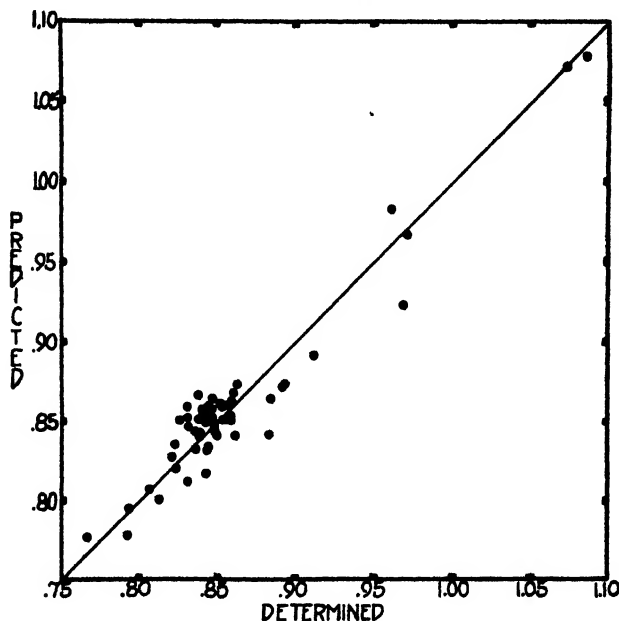


FIG. 2. Agreement of determined values of equivalent vapor pressure with those predicted from total base, non-protein nitrogen, and protein by Equation 1. The values are expressed as gm. per cent of sodium chloride.

of the variables, namely protein, was omitted. For further simplification and for reasons which will appear later the units selected for expressing the base and nitrogen values were those in common usage and not those which refer the concentrations to serum water; that is, total base was expressed as milli-equivalents per liter of serum and non-protein nitrogen as mg. per 100 cc. of serum. The argument for so simplifying the procedure follows: If a constant volume of serum is conceived as having a fixed con-

tent of total base and non-protein nitrogen, there are two theoretical effects on osmotic pressure of varying the protein concentration. One effect results from the fact that increases in protein must be attended by decreases in serum water, so that no change in volume will occur; the decrease in serum water will increase the molal concentration of the other solutes and so lead to an increase in osmotic pressure. Another effect arises from the circumstance that increases in protein must be associated with de-

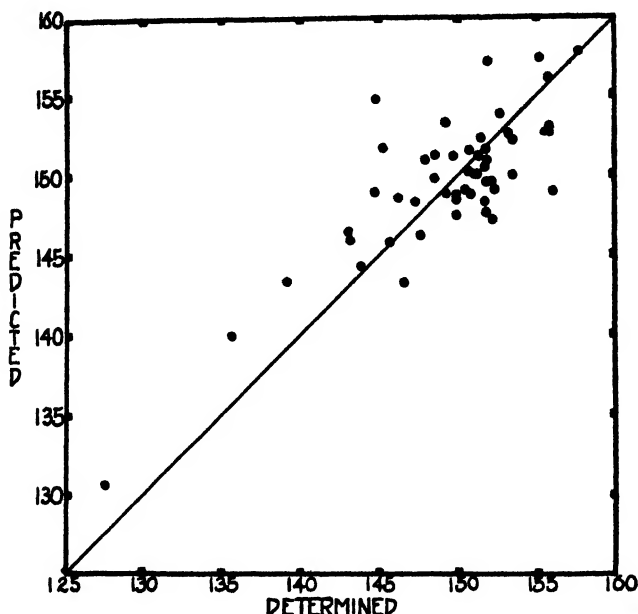


FIG. 3. Agreement of determined values of total base with those predicted from equivalent vapor pressure, non-protein nitrogen, and protein by Equation 2. The values are expressed as milli-equivalents per liter.

creases in some of the osmotically active anions, chiefly chloride and bicarbonate, in order to maintain electrolyte balance; since the protein itself is osmotically inactive, this effect leads to a decrease in osmotic pressure. A further decrease in osmotic pressure results from the effect of protein on the ionization of calcium and magnesium. In our theoretical system there are, then, two effects from augmenting the protein, one tending to raise and the other to lower the osmotic pressure. To some extent

at least the effects must cancel and to this same extent must the osmotic pressure be independent of variations in protein when the cation and non-electrolyte concentrations are given in relation to volume of serum rather than gm. of serum water. The result of correlating the data in this way follows:

$$\begin{aligned} R_{V(b, n)} &= 0.9621 \\ V &= 0.004012b + 0.001194n + 0.20683 \\ P.E. (est. V) &= 0.01055 \end{aligned} \quad (4)$$

It appears both from the coefficients of multiple correlation and from the probable errors of estimate that Equation 4 predicts the equivalent vapor pressure almost as accurately as Equation 1. The agreement between determined values of equivalent vapor pressure and those predicted by Equation 4 is represented graphically in Fig. 4.

Relationship in Serum between Total Base and [Equivalent Vapor Pressure and Non-Protein Nitrogen]—When the procedure of the preceding paragraph is altered so that total base is treated as the dependent variable, the following relationships emerge:

$$\begin{aligned} R_{b(V, n)} &= 0.8128 \\ b &= 164.34V - 0.1989n + 17.16 \\ P.E. (est. b) &= 2.135 \end{aligned} \quad (5)$$

It is seen again that Equation 5 predicts the total base almost as accurately as Equations 2 and 3. The agreement between found and predicted values is shown in graph form in Fig. 5.

Comment

The relationship expressed by Equation 1 and represented graphically in Fig. 2 between the equivalent vapor pressure of serum and several of its solutes demonstrates conclusively that the rate of evaporation of serum under standard conditions of environment varies directly with the concentration of its solutes. The form of the equation, in which positive contributions to the equivalent vapor pressure are made by the total fixed base and the non-protein nitrogen and a negative effect is exerted by protein, strongly suggests that the same property of serum which determines the vapor pressure and osmotic pressure also determines the equivalent vapor pressure. It is clear that there are no

large random variations in the equivalent vapor pressure which cannot be explained by fluctuations in the chemical composition of the sera. Much of the variation which was recorded is undoubtedly traceable to the combined effect of errors of measurement. However, the method of statistical analysis does not and cannot exclude the possibility of some constant influence on the rate of evaporation of serum which would render all results for equivalent vapor pressure consistently either too low

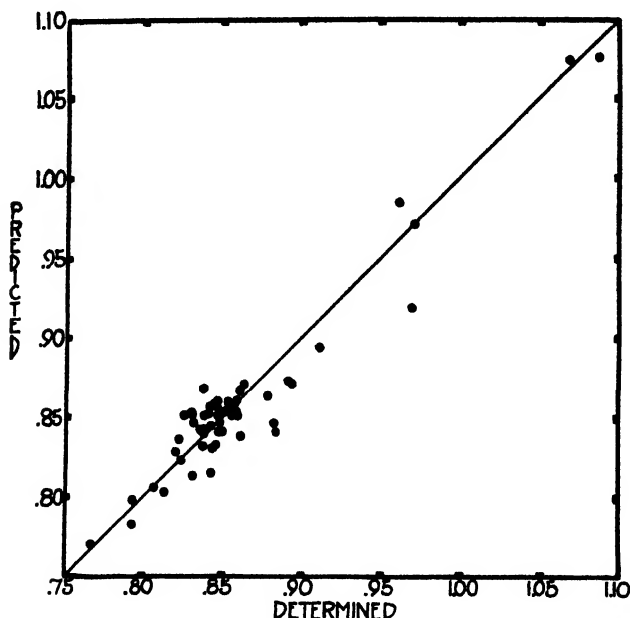


FIG. 4. Agreement of determined values of equivalent vapor pressure with those predicted from total base and non-protein nitrogen by Equation 4. The values are expressed as gm. per cent of sodium chloride.

or too high when translated into terms of vapor pressure or osmotic pressure. Such an effect might conceivably result if the surface of the evaporating sample were always coated with a monomolecular layer of protein molecules; if a layer of this type interferes with the rate of evaporation, the interference will clearly be independent within wide limits of the concentration of protein in the serum. As yet no direct means of measuring the osmotic pressure of serum is available, nor does a method exist

for direct determination of vapor pressure which is accurate enough to throw light on this point. For the time being, therefore, it appears reasonable to think that the thermoelectric procedure measures that same property of serum which determines its osmotic pressure. In so far as this is true the equations which have been developed should prove useful when estimates of the osmotic pressure of sera are wanted. The estimates so obtained will be in the form of equivalent gm. per cent of sodium chloride,

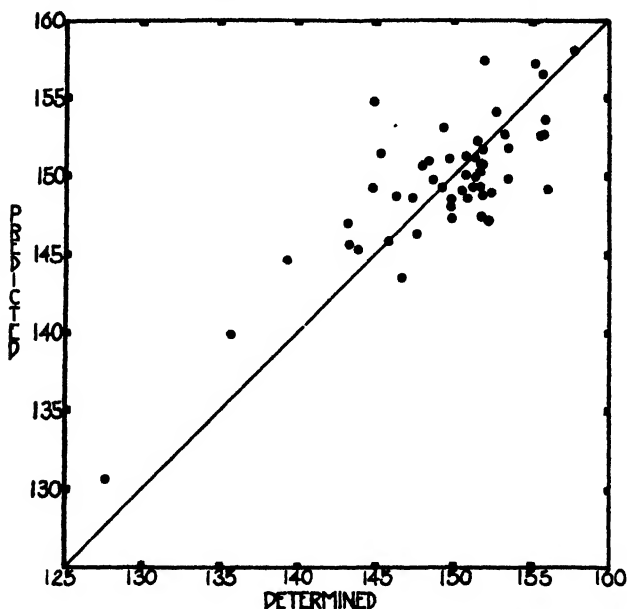


FIG. 5. Agreement of determined values of total base with those predicted from equivalent vapor pressure and non-protein nitrogen by Equation 5. The values are expressed as milli-equivalents per liter.

which is to say that the osmotic pressure of the serum under investigation is the same as that of a solution of sodium chloride of the estimated concentration. This result is easily expressed in the more conventional terms of mm. of mercury at 25° by multiplying by the factor 5901.²

² Calculated in the manner outlined by Findlay (10). The value for the depression of freezing point used in computing the osmotic activity coefficient of the standard 0.92 per cent solution of sodium chloride was taken

The equations for predicting base are of limited value, since the apparatus for measuring equivalent vapor pressure is not widely distributed. They have, however, been serviceable in this clinic when with individual patients it was desired to construct rapidly a picture of the electrolyte partition of the serum. For this purpose measurement of specific gravity provides a rapid means of estimating the protein content (11). In an investigation preliminary to the present study a regression equation was calculated for predicting total base from the sum of bicarbonate, chloride, and protein ions. It is interesting that Equations 2 and 3 and Equation 5 predict the total base more accurately than was possible by the preliminary method. This is true in spite of the fact that sera with presumptively high values for undetermined acids were not included in either series.

It may be well to state that the values dealt with in this paper are not identical with that property of serum which renders it isotonic with respect to red blood corpuscles. Examination of the data in Table I indicates that marked elevation of equivalent vapor pressure occurs when the non-protein nitrogen of serum is high. Such sera do not cause shrinkage of red cells because the cell membranes are freely permeable to urea and probably to other molecules in the non-protein nitrogen complex. A question may be raised concerning the validity of regarding the osmotic pressure due to protein as negligible when the total osmotic pressure is being recorded. The so called colloid osmotic pressure of protein, as usually measured, is in the neighborhood of 0.5 per cent of the total pressure, hence within the error of measurement of equivalent vapor pressure. Moreover, it must be remembered that colloid osmotic pressure is always measured in a system in which a semipermeable membrane is utilized and that a part, about one-fifth (12), of the recorded pressure results from the Donnan effect in producing unequal distribution of the other ions. This portion of the colloid osmotic pressure does not exist as a property of protein under the conditions of measurement of equivalent vapor pressure.

from the "International critical tables," volume 4. The coefficient is 1.855. Calculation of the factor involves the following steps: mm. of Hg osmotic pressure = $V \times 10 \times 1.855 \times 22.412 \times 760 \times (273 + 25) + 58.45 \times 273 = 5901V$.

SUMMARY

The method devised by Hill for measuring the vapor pressure of whole blood is basically a measure of the rate of evaporation of the sample. Arguments against the validity of regarding such measurements as functionally related to vapor pressure and osmotic pressure are considered. To facilitate discussion, that concentration of a solution of sodium chloride which evaporates at the same rate as an unknown sample of serum or blood is defined as the "equivalent vapor pressure."

In 53 samples of human serum measurements were made of equivalent vapor pressure, and of the concentrations of total base, non-protein nitrogen, and protein. From the data the regression equation was computed which permits the prediction of equivalent vapor pressure from the three solutes. Both the accuracy of the predictions and the form of the equation lend support to the assumption that the rate of evaporation of serum is determined by the same property which also determines vapor pressure and osmotic pressure.

From the same data the regression equation was computed which permits the prediction of total fixed base from measurements of equivalent vapor pressure, non-protein nitrogen, and protein. The estimate of total base has a probable error of ± 2.1 milli-equivalents per liter.

Similar regression equations for predicting equivalent vapor pressure and total base were computed from the same data when one of the variables, protein, was omitted. By expressing the concentrations of total base and non-protein nitrogen in units which relate them to volume of serum rather than to gm. of serum water, some compensation was made for the loss in accuracy of prediction which would have resulted from simple omission of the protein variable.

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THE SEXUAL VARIATION IN CARBOHYDRATE METABOLISM

VIII. THE RATE OF ABSORPTION OF GLUCOSE AND OF GLYCOGEN FORMATION IN NORMAL AND ADRENALECTOMIZED RATS*

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For some time it has been clearly recognized that the metabolism of carbohydrate and fat shows a considerable variation with sex. Not only is the extent of ketonuria much greater in women than in men (1) but similar differences are noted in the artificial ketonuria produced in rats and guinea pigs by the administration of sodium acetoacetate (2). Moreover, it has recently been demonstrated that the fasting ketonuria developed in rats previously on high fat diets is much greater in the female (3).

That such variations in the excretion of ketone bodies are referable to differences in carbohydrate metabolism was shown by Deuel, Gulick, Grunewald, and Cutler (4) who demonstrated that the liver glycogen values of rats fasted for as long as 3 days after glucose were invariably higher in the males than in the females. In this early work, however, no differences were found in the level of liver glycogen of unfasted male and female rats. However, in a recent study (3) of this in a large group of rats on various high fat diets it was noted that the height of liver glycogen in the female averaged only about 60 per cent of that in the males.

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Some of these data were reported at a meeting of the Society for Experimental Biology and Medicine at La Jolla, California, February 27, 1937.

Such a sexual difference was also found in rats previously fed on the high carbohydrate stock diet.

If the level of liver glycogen in the female continually is present only to the extent of approximately 60 per cent of that in the male, there must be a smaller amount of ingested carbohydrates which are capable of transformation to glycogen. Such a discrepancy has not been noted, however. Cori (5) in his classical experiments on the rate of glycogen formation after various sugars employed only male rats. Had female rats been used, very different results would have been noted.

Such sex differences are probably largely traceable to some ovarian hormone other than theelin. Not only is the liver glycogen significantly higher in ovariectomized rats fasted 48 hours than in their normal sisters (6), but also the level of ketonuria induced in such animals by the administration of sodium acetoacetate is lower for as long as 5 days, the period of the experiments (7). Later (8) it was postulated that such a discrepancy might be referable to the ketogenic hormone of the anterior lobe of the pituitary, inasmuch as this hormone provoked as great a response in male as in female rats and as much in the spayed as in the unoperated female. We suggested that the greater ketonuria in the female was probably associated with a greater stimulation of the ketogenic hormone of the pituitary in this sex, which must be caused by an ovarian hormone.

That such a ketogenic hormone may exert its activity through the adrenal cortex seems possible from the recent reports of various laboratories. Thus, Long and Lukens (9) found a reduction in the urinary ketones in depancreatized cats following adrenalectomy, while Evans (10) reports a similar effect in phlorhizinized rats. MacKay and Barnes (11) report a lower ketonuria in fasting adrenalectomized rats previously on a high fat diet than in normal rats so treated. These authors (12) have also reported that the removal of the adrenals abolishes the ketosis of late pregnancy in the rat. All of these results would seem to indicate that the sex differences in ketosis might be associated ultimately with the action of a cortical hormone.

The fact that the liver glycogen of adrenalectomized animals of various species rapidly falls practically to zero values after short periods of fasting irrespective of the sex of the animal (13) might

also suggest that the sex difference in carbohydrate metabolism is abolished by adrenalectomy. Parkins, Hays, and Swingle (14) believe, however, that such effects on carbohydrate deposition are not concerned directly with the functions of the adrenal cortex but occur because of the profound toxicosis resulting from lack of the cortical hormone.

In the present study a comparison of the rate of glycogen formation in the liver was made in normal male and female rats when glucose was administered in an excess over a period of hours. Such results were compared with those on adrenalectomized animals. Likewise, the rate of absorption from the gastrointestinal tract was studied, since it has been shown by Bennett (15) that absorption of glucose is slower in hypophysectomized rats, while Buell, Anderson, and Strauss (16) noted that the absorption of lactic acid is retarded in adrenalectomized rats. Leloir (17) has noted that the adrenalectomized dog has a decreased ability to store glycogen.

On the other hand, Samuels, Schott, and Ball (18) found that, while there was a decrease in glucose tolerance in adrenalectomized rats, the administration of Rubin and Krick's solution (19) delayed its onset and adrenal cortex extracts prevented the alteration entirely.

Methods

Rats 75 to 120 days old, from our colony, were employed. The experiments on the adrenalectomized rats were made 12 to 20 days after the operation. The animals were maintained in the interim on Rubin and Krick's solution (19) for drinking and on the usual stock diet, in a room kept at 25–27°. The operated animals remained in excellent nutritional condition throughout as demonstrated by the fact that practically no adrenalectomized animals died during the period preparatory to the tests reported here. On the other hand, twenty rats which were adrenalectomized and kept under similar conditions except that the Rubin-Krick solution was replaced with water were all dead within 17 days.

The normal rats were fasted 48 hours prior to the glucose feeding, while the adrenalectomized ones were only fasted overnight (16 hours). The difference in the time intervals employed was due to the rapid depletion of liver glycogen in the operated

animals. Approximately 600 mg. of glucose per 100 gm. of body weight were given by stomach tube at the start of the test. Additional doses of 500 mg. per 100 gm. of body weight were given the 3rd, 5th, and 7th hours for the 4, 6, and 8 hour tests. Approximately 50 per cent glucose solutions were employed. In no cases did diarrhea result from these doses of glucose. The animals were killed under amytal anesthesia. Glycogen was determined by the method of Good, Kramer, and Somogyi (20).

The gastrointestinal tract was removed after the esophagus was clamped. It was finely macerated with scissors and boiled for a minute with 200 cc. of water. The proteins were precipitated by Somogyi's reagent (10 per cent $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.50 N NaOH) and made up to a liter after removal of most of the pieces of gut. From the filtrate we have been able to recover approximately 99 per cent of sugar administered as described above to a living rat, when the gastrointestinal tract was immediately removed. Although our usual practise was to carry out the analyses immediately on the gut filtrates, the glucose in the filtered solution keeps perfectly in the refrigerator overnight without preservative. The completeness of the adrenalectomy was always confirmed at autopsy.

Results

The summary of the experiments showing the glycogen deposited in the liver of the normal and adrenalectomized rats is given in Table I. The level of liver glycogen in the fasted normal rats as well as in each group after the feeding of glucose is significantly higher in the male than in the female rats. On the other hand, no sex variation was noted in the practically blank value for the level of liver glycogen in the adrenalectomized rats fasted 16 hours. However, the values obtained at various periods after the administration of glucose again demonstrate that the glycogen formation is greater in the males. With the exception of the 8 hour group, all differences are significant.

In Table II is recorded a summary of the values on the rate of absorption of glucose from the gastrointestinal tract. The rate of absorption is fairly constant, averaging 132 mg. per 100 gm. of body weight in the normal males and 156 mg. per 100 gm. in the normal females. In the adrenalectomized rats the absorp-

tion was somewhat higher. The mean value for the males and females respectively was 143 and 161 mg. per 100 gm. of rat. In both cases the average absorption rate is significantly different for the sexes. The level of reducing materials in the gut of the fasting animals was insignificant compared with the quantities absorbed, so no correction has been made for it.

TABLE I

Liver Glycogen of Normal and Adrenalectomized Rats Previously Fasted for Various Periods after Receiving an Excess of Glucose by Stomach Tube

Experimental condition	Liver glycogen of fasted controls	Total liver glycogen				Glycogen deposited*			
		2 hrs.	4 hrs.	6 hrs.	8 hrs.	2 hrs.	4 hrs.	6 hrs.	8 hrs.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Normal males	0.59 (15)	2.16 (10)	3.64 (10)	5.13 (10)	6.22 (10)	1.57	3.05	4.54	5.63
“ females	0.19 (15)	1.32 (10)	2.44 (10)	3.30 (9)	4.78 (10)	1.13	2.25	3.11	4.59
M.D./P.E.M.D.†	7.02	5.25	5.00	8.43	7.38				
Adrenalectomized males	0.03 (17)	1.17 (24)	3.00 (22)	4.23 (19)	5.17 (18)	1.14	2.97	4.20	5.14
Adrenalectomized females	0.04 (14)	0.92 (15)	2.44 (13)	3.64 (14)	4.65 (9)	0.87	2.39	3.59	4.60
M.D./P.E.M.D.†		3.25	5.88	4.36	2.14				

The figures in parentheses represent the number of experiments in the preceding average.

* The value of the total glycogen in the liver less that obtained for the fasting controls.

† Mean difference to probable error of mean difference of the average value for males as compared to females. When this value exceeds 3, the results are considered statistically significant.

DISCUSSION

The data demonstrate that there is no marked variation in the absorption and metabolism of glucose in the adrenalectomized rats when the observations are made on animals sufficiently long after the operation to avoid the effect of postoperative shock and before toxic symptoms resulting from the absence of the cortical hormone are noted. Such experiments have been made in the

present investigation 12 to 20 days following the operation. It should also be noted that normal results can only be expected when the salt balance is kept as nearly physiological as possible by the administration of a salt solution as suggested by Rubin and Krick. In no case in the present tests did the animals appear to have a toxic condition caused by the absence of the cortical hormone. There is no question that the rate of glucose absorption and of glycogen formation may be far below that in normal animals when the toxicosis sets in. As evidence of this

TABLE II
Rate of Absorption of Glucose from the Intestines of Normal and Adrenalectomized Rats

Experimental condition	Reduction of gastro-intestinal tract of controls	Glucose absorption, mg. per 100 gm. per hr.					M.D./P.E.M.D., male compared with female	M.D./P.E.M.D., normal compared with adrenalectomized
		2 hrs.	4 hrs.	6 hrs.	8 hrs.	Average		
Normal males	mg. 6.7 (8)	128 (5)	117 (6)	157 (6)	128 (8)	132 (25)	6.33	2.93
“ females	3.2 (10)	146 (6)	152 (6)	171 (5)	156 (9)	156 (26)		
Adrenalectomized males	4.3 (15)	132 (16)	143 (16)	151 (13)	149 (9)	143 (54)	5.13	
Adrenalectomized females	5.4 (8)	154 (6)	150 (6)	161 (8)	175 (7)	161 (27)		

The figures in parentheses represent the number of experiments in the preceding average.

we have found entirely different results in nitrogen and ketone body excretion in rats suffering from advanced adrenal deficiency than in other operated animals which remained in apparently normal condition. Periods of oliguria may occur for more than 24 hours prior to death, during which temperature regulation and circulation are obviously abnormal. It is apparent that under such conditions one is dealing not alone with the effect of the adrenal hormones but also with other secondary alterations in metabolism which might change the carbohydrate metabolism significantly, even in the presence of the adrenal.

The present experiments demonstrate that the extent of glycogen formation following the administration of large amounts of glucose is greater in the male than in the female. That such a variation is not the result of a more rapid rate of formation is indicated by the fact that the variation is still noted after an 8 hour interval. The difference must be traceable to a greater oxidation of glucose during the absorption period in females or to a more ready conversion to fat. This discrepancy cannot be due to a slower rate of absorption, for it is demonstrated here that it is actually more rapid in the female rats than in the males. One is unable to explain the sex differences in the liver glycogen level of unfasted rats which we have noted after a variety of diets (3) or in sexually mature rats (21) in any other way than in a difference in the degree of formation in the two sexes.

The rate of glycogen deposition was practically identical in the adrenalectomized and normal females. In the experiments on males, there was a slightly lower level of glycogen deposition in the operated animals after the longer time intervals. This difference is not significant, however. Therefore, it would appear that there is no abnormality in glycogen deposition due specifically to removal of the adrenal glands, since both absolute level and sex difference are maintained. Also the changes in glucose tolerance noted in adrenalectomized animals (18) would seem to be due to the secondary changes in the liver which follow severe adrenal deficiency, since those conditions which maintain the normal glycogenic function also preserve the normal tolerance.

The rate of absorption is somewhat lower than the values given by Cori (5) which varied from averages of 196 to 161 mg. per 100 gm. of body weight for the experiments in which glucose was given in 50 per cent solution and from 184 to 143 mg. for those in which this sugar was administered in 80 per cent solution. It should be noted that low values in the present tests are not to be traced to a failure to recover the glucose completely from the gut; on the contrary, since absorption is calculated from the difference between the amount fed and that recovered, any loss in the latter procedure should give an apparently greater rate of absorption. We have found that more consistent results can be obtained by analysis of the whole gut than by employing gut washings. In harmony with the results of Cori (5), there is no

definite trend indicating a variation in the rate of absorption over longer periods, although our results were obtained on rats receiving repeated doses of glucose at 2 hour periods rather than a single large quantity.

SUMMARY

In normal and adrenalectomized rats there is a sex difference in the extent of glycogen formation in the liver following the administration of glucose. The glycogen deposition for periods as long as 8 hours, during which maximum quantities of glucose are being absorbed, is quite similar for the normal and operated rats.

The rate of glucose absorption is significantly higher in the female than in the male, both in the normal and adrenalectomized rats. Moreover, there is no evidence that the rate of glucose absorption was decreased after adrenalectomy.

It is concluded that the adrenal cortex does not directly alter the ability of the rat to absorb glucose or to deposit glycogen. Also it is not the endocrine gland ultimate responsible for the sex difference in carbohydrate metabolism.

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THE SEXUAL VARIATION IN CARBOHYDRATE METABOLISM

IX. THE EFFECT OF AGE ON THE SEX DIFFERENCE IN THE CONTENT OF LIVER GLYCOGEN*

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The liver glycogen of normal adult male rats remains at a constantly higher level than that of the females during periods of fasting up to 3 days (1). Likewise, the extent of ketonuria which develops in fasting female rats when sodium acetoacetate is fed (2) or in such animals following a high fat diet which results in a deposition of fat in the liver (3) is continually higher than in the males subjected to similar experimental conditions. Since differences in glycogen level (4) as well as in the degree of ketonuria (5) are abolished by ovariectomy, it seems probable that both of these phenomena are traceable to some hormone produced in the ovary which may alter the course of carbohydrate metabolism.

Although in our original report (1) we were unable to demonstrate the fact that there is a sex difference in the amount of liver glycogen in *unfasted* animals, it has been recently shown (3) that a marked sex variability in liver glycogen invariably occurs in unfasted rats receiving a variety of high fat diets or our stock diet. The level of liver glycogen in the females averaged only about 60 per cent of that in the livers of males.

If the alteration in carbohydrate metabolism is related to the secretions of the sex glands, it seems probable that differences in glycogen level would be abolished before maturity and might also be minimized in old age. In fact, we have elsewhere suggested that the inability to show sex differences in unfasted animals which

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were obtained from commercial sources may have been due to their age.

In the present study we have compared the level of liver glycogen in unfasted male and female rats of various ages which have been continuously maintained on the same dietary régime. Not only should such data give the variations in glycogen storage with the age of the animals, but they should also demonstrate whether sex differences in carbohydrate metabolism are absent before maturity and whether they are abolished in senescence.

TABLE I

Summary of the Average Values of Liver Glycogen in Normal Unfasted Male and Female Rats of Various Ages

Age	No. of experiments		Body weight		Liver weight		Liver		Liver glycogen		
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	M.D. P.E.M.D.
days			gm.	gm.	gm.	gm.	per cent body weight	per cent body weight	per cent	per cent	
26-29	18	22	61	57	3.29	3.11	5.38	5.42	7.01	7.02	
39-40	9	9	104	93	6.06	5.51	5.82	5.92	8.42	7.94	1.61
41-45	13	12	121	105	6.82	6.02	5.63	5.73	7.32	6.56	3.14
54-55	20	20	155	123	7.65	6.08	4.93	4.94	6.05	5.51	3.42
73-76	10	10	200	149	8.32	6.43	4.25	4.31	4.25	2.59	9.48
88-90	5	6	235	167	9.06	6.36	3.87	3.78	4.34	1.79	5.12
mos.											
17-18	19	20	343	249	11.62	8.60	3.39	3.45	3.91	3.80	
21-24	20	19	359	250	11.82	8.80	3.29	3.52	4.16	3.93	

* Mean difference to probable error of mean difference of the average result on males compared with the mean on females. When this value exceeds 3, the results are considered significant.

The male and female rats employed in the various groups were composed of litter mates. The animals were all killed over a brief period in the spring, which should avoid any differences due to seasonal variation. Likewise, they were sacrificed early in the morning (8 to 10 a.m.) which would prevent discrepancies traceable to a possible diurnal variation (6). Glycogen was determined by the method of Good, Kramer, and Somogyi (7). Amytal was employed as an anesthetic. The essential results are summarized in Table I.

In the youngest rats the level of liver glycogen is identical in the two sexes. The value is very high compared with that in the normal unfasted adult but only slightly greater than that reported by Corey (8) for rats at term. This investigator noted that the liver glycogen gradually rose from a level of 1 per cent in the smallest fetuses to one of 6 per cent obtained on new born rats. In the present tests the highest values obtained on 39 to 40 day-old males (8.42 per cent) gradually dropped to a level of approximately 4 per cent (73 to 75 days of age). With the exception of the youngest and oldest groups, the values on the females were consistently lower than those on the males. The difference is significantly lower starting with the 41 to 45 day-old group. In this series of tests the minimum level of liver glycogen obtains in females 3 months old (1.79 per cent), in which it is only 41 per cent of that of the males. In the old females (17 to 24 months), the liver glycogen has increased to values practically identical with those of the male. The factor which causes the sex difference in carbohydrate metabolism of the younger animals is apparently not operating in the old females.

The curve for the difference in liver glycogen shows that this alteration is apparently referable directly to the sex glands. The results exclude the adrenals, the pituitary, and thyroid glands as factors concerned in this regulation inasmuch as they do not show such variations before maturity and in senescence as we have observed here. Also the decreased level obtained in the female on sexual maturity which disappears in old age fixes the sex difference on an inhibitory glycogenic effect probably caused by the ovary rather than on a stimulatory effect on glycogenesis traceable to a testicular secretion in the male.

Although the food intake was not determined prior to the tests, the animals were all healthy rats which had eaten as much of our stock diet as desired. According to Wang (9), the food consumption during the early life (20 to 50 days) is much higher on a kilo or surface area basis than during the later periods. Such a period of high food intake corresponds with the time of high liver glycogen. The higher rate of food intake may continue to the 90th day with the males. However, the amount calculated on the basis of body weight shows that the males ate on an average only 94.6, 97.2, 77.0, and 74.6 per cent respectively of that in-

gested by the females for the four 10 day intervals from 50 to 90 days of age. That a higher food consumption by the males is not responsible for the differences between the sexes in glycogen store is also shown by the fact that we have repeatedly noted similar sex differences in the liver glycogen of unfasted rats (about 3 months of age) on various high fat diets where no variations in food intake on the weight basis could be shown (3). During the second phase, the food intake is somewhat higher on a weight or surface area basis in the female than in the male. The per cent of the body weight which the liver makes up is abnormally high in the young rats, reaching the highest level in the 6 week group. From this it gradually falls to a minimum value of 3.29 per cent in the old males and 3.45 per cent in the old females.

SUMMARY

The level of glycogen present in the livers of unfasted rats is highest at 39 to 40 days, at which time it exceeds 8 per cent. From this level it gradually drops to a value of about 4 per cent which is found in male rats 75 days of age. Approximately the same value was noted in rats 19 to 24 months old. There was no sexual difference noted in the liver glycogen of rats 26 to 29 days of age nor in the old rats (17 to 24 months). In the other groups the level of liver glycogen in the females is constantly lower than that of the males. The liver glycogen reached a minimum value in the females which were 3 months old.

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THE CHEMICAL ACTIVATION OF STEROLS

III. THE CHEMICAL ACTIVATION OF CHOLESTEROL*

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The chemical antirachitic activation of sterols originated with the discovery of Bills (1) that cholesterol could be activated to a slight extent by heating a carbon tetrachloride solution of cholesterol with floridin. Later Yoder (2) found that cholesterol could also be converted into an active product by treatment with sulfur trioxide or with a mixture of sulfuric acid and acetic anhydride. In a study of the activation with sulfuric acid-acetic anhydride (3), it has been shown that a specific relationship probably exists between chemical configuration and chemically activatable cholesterol or cholesterol derivatives. The activation of cholesterol with sulfuric acid-acetic anhydride was investigated in an attempt to determine the mechanism of the reaction.

It has been found (3) that the provitamin D of cholesterol is not the precursor of the active substance produced by the action of sulfuric acid-acetic anhydride on cholesterol. The possibility of the provitamin D of heated purified cholesterol being the precursor of the active substance was investigated and the effect of ultraviolet irradiation on the potency of the reaction product was determined.

EXPERIMENTAL

The effect of various proportions of acetic anhydride and sulfuric acid on the activation of cholesterol was studied. In a series of 50 cc. Erlenmeyer flasks were placed 0.001 mole of dry cholesterol, 4 cc. of glacial acetic acid (99.5 per cent), and various

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quantities of acetic anhydride (98 per cent) and concentrated sulfuric acid (95 per cent). The reaction mixtures were heated to 90° , stoppered with a cork, and then the heating was continued in an oven at $85-90^{\circ}$ for 3 hours. The reaction products were concentrated and aliquots of the ether-alcohol solutions of the residues containing the equivalent of 6 mg. of the original cholesterol were tested biologically with rats for antirachitic activity. It was found that the maximum antirachitic potency produced by this procedure was obtained when the molar proportions were 0.0025 mole of acetic anhydride and 0.002 mole of sulfuric acid per 0.001 mole of cholesterol.

The effect of time during which 0.001 mole of cholesterol was heated in 4 cc. of glacial acetic acid solution at $85-90^{\circ}$ with 0.0025 mole of acetic anhydride and 0.002 mole of sulfuric acid was investigated. It was found that the maximum potency was obtained in about 3 hours and that the potency was about the same when the reaction mixture was heated any length of time between 3 hours and 40 hours.

The effect of temperature in the production of antirachitically active material from cholesterol also was studied. It was found that the product obtained by a 3 hour treatment of cholesterol with sulfuric acid-acetic anhydride at room temperature was inactive at the 24 mg. level, slightly active if treated at 55° , and most potent when produced at about $85-90^{\circ}$. Although the product obtained when the temperature was maintained at 24° for 3 or 24 hours failed to induce noticeable calcification when fed to rachitic rats at the 24 mg. level, it was found that when the reaction mixture which had stood at 24° for 3 or 24 hours was then heated at $85-90^{\circ}$ for 3 hours the product was found to possess about the same antirachitic activity as if the heating had been carried out at once.

Furthermore, it was observed that sulfur dioxide was slowly evolved when the reaction mixture was allowed to stand at room temperature for a few hours and the evolution of a trace of carbon dioxide was detected when the reaction mixture had stood for 40 hours. Both sulfur dioxide and carbon dioxide were evolved when the reaction mixture was heated at $85-90^{\circ}$ for 3 hours.

The evolution of sulfur dioxide was detected by passing nitrogen through the reaction mixture and into a barium hydroxide solu-

tion. The white precipitate which separated from the barium hydroxide solution was removed by filtration and dissolved in dilute hydrochloric acid; the odor of sulfur dioxide was unmistakable. Bromine water was added to the hydrochloric acid solution and a white precipitate was formed, demonstrating that sulfur dioxide was formed in the reaction mixture. The quantity of evolved sulfur dioxide was determined and the evolution of carbon dioxide was detected by passing nitrogen through the reaction mixture and collecting the sulfur dioxide in dilute hydrogen peroxide and the carbon dioxide in barium hydroxide solution. The nitrogen was bubbled through a barium hydroxide solution and passed through a calcium chloride tube before it was passed into the reaction mixture. The stream of gas from the reaction mixture was bubbled through 50 cc. of a 2 per cent hydrogen peroxide solution to collect the sulfur dioxide and then through 50 cc. of a saturated barium hydroxide solution to collect the carbon dioxide. The system was flushed out with nitrogen and then a slow stream of nitrogen was passed through while the reaction mixture in a 50 cc. flask was heated at 85–90° in a water bath. The hydrogen peroxide solution acidified with 1 cc. of 6 N hydrochloric acid was heated to boiling, and a solution of barium chloride was added dropwise with stirring. After cooling, the barium sulfate was filtered, ignited, and weighed. The white precipitate in the barium hydroxide solution was soluble in acetic acid and no precipitate was obtained by the addition of bromine water to a hydrochloric acid solution of the precipitate, demonstrating that the white precipitate was barium carbonate.

The observation that heat is essential in the production of maximum activity led to a study of the sulfuric acid-acetic anhydride reagent. Below 0° sulfuric acid reacts with acetic anhydride to form acetylsulfuric acid which, if warmed, is converted to sulfoacetic acid (4). Sulfoacetic acid can be prepared by heating a mixture of sulfuric acid and an excess of acetic anhydride to 80° to the disappearance of free sulfuric acid (5, 6) and by treating acetic acid with fuming sulfuric acid (7).

Cyclohexene and camphor can be converted into sulfonic acids by means of a mixture of sulfuric acid and acetic anhydride (acetylsulfuric acid). Friese (8) found that cyclohexene in acetic acid solution could be converted into *o*-cyclohexanolsulfonic acid

by the action of a molar equivalent of sulfuric acid and an excess of acetic anhydride at -20° and then warmed up to room temperature. When *d*-camphor (1 mole) is treated with a well cooled mixture of sulfuric acid (1 mole) and acetic anhydride (2 moles), it is converted into *d*-camphor-10-sulfonic acid (9, 10), whereas it yields *dl*-camphor-8(or 9)-sulfonic acid by the action of either fuming sulfuric acid or chlorosulfonic acid (11). A sulfonic acid group enters a (different) methyl group in these camphorsulfonic acids.

Carbon dioxide was evolved when a solution of sulfoacetic acid prepared from 0.002 mole of sulfuric acid and 0.005 mole of acetic anhydride, dissolved in 4 cc. of glacial acetic acid, was heated at $85-90^{\circ}$ for 3 hours. Melsens (7) found that carbon dioxide was evolved when heat was applied during the preparation of sulfoacetic acid by the action of fuming sulfuric acid on acetic acid. In addition to carbon dioxide a trace of sulfur dioxide was evolved when a solution of 0.002 mole of sulfuric acid or a mixture of 0.002 mole of sulfuric acid and 0.0025 mole of acetic anhydride dissolved in 4 cc. of glacial acetic acid was heated at $85-90^{\circ}$ for 3 hours.

Various Reagents

The effect of sulfuric acid, sulfoacetic acid, fuming sulfuric acid, and chlorosulfonic acid in imparting antirachitic activity to cholesterol was investigated. Data obtained to determine the antirachitic activity of the products prepared by the action of various reagents on cholesterol are presented in Table I.

The action of the sulfuric acid on cholesterol in the ratio of 2 moles per mole was studied. In a 50 cc. Erlenmeyer flask were placed 0.001 mole (0.386 gm.) of dry cholesterol, 4 cc. of glacial acetic acid, and 0.002 mole (0.112 cc.) of concentrated sulfuric acid. The reaction mixture was heated at $85-90^{\circ}$ for 3 hours, during which time a color change through violet to green to a brownish purple color was observed, a solid separated, and sulfur dioxide and carbon dioxide were evolved. After the reaction product was concentrated, the residue was found to be less active than the product which has been produced consistently by the action of the sulfuric acid-acetic anhydride reagent.

Cholesterol was treated with sulfuric acid and the reaction

product was heated with acetic anhydride. A mixture of 0.001 mole by dry cholesterol, 4 cc. of glacial acetic acid, and 0.002 mole of concentrated sulfuric acid was heated at 85–90° for 3 hours. Then 0.0025 mole (0.24 cc.) of acetic anhydride was added and the reaction mixture was heated an additional 3 hours, during which time no change in appearance of the reaction mixture was observed. This reaction product was about as potent as that produced by the action of sulfuric acid.

TABLE I

Antirachitic Activation of Cholesterol by Heat Treatment with Various Reagents

The line test gave average plus values of 2.5 for each reagent. A 2 plus value here denotes approximately 5 vitamin D units, United States Pharmacopœia XI (12).

Reagents used	Quantity of product fed per rat*	No. of rats
	mg.	
H ₂ SO ₄	12	5
" then Ac ₂ O	12	5
Ac ₂ O, then H ₂ SO ₄	6	5
HO ₂ SCH ₂ COOH	12	5
Ac ₂ O, then HO ₂ SCH ₂ COOH	4	6
HOSO ₂ OSO ₂ OH	6	5
ClSO ₂ H	6	5
H ₂ SO ₄ ·Ac ₂ O	6	4

* Based on the weight of the original cholesterol.

The action of acetic anhydride followed by sulfuric acid was studied. A mixture of 0.001 mole of dry cholesterol, 4 cc. of glacial acetic acid, and 0.0025 mole of acetic anhydride was heated at 85–90° for 3 hours. Then 0.002 mole of concentrated sulfuric acid was added and the reaction mixture was heated an additional 3 hours, during which time the colorless solution changed through a green to a brown color, sulfur dioxide and carbon dioxide were evolved, and a resin separated on the sides of the flask. This product was as active as that produced by the action of the sulfuric acid-acetic anhydride reagent.

The action of sulfoacetic acid on cholesterol was investigated. A sample of sulfoacetic acid was prepared in a cut-off sample tube by heating a mixture of 0.002 mole of sulfuric acid and 0.005 mole of acetic anhydride at 85° for 3 minutes. The mixture was stirred before and after heating with a short stirring rod. The tube containing the sulfoacetic acid was dropped into a flask containing 0.001 mole of dry cholesterol and 4 cc. of glacial acetic acid and the sulfoacetic acid on the stirring rod was washed into the flask by means of a few drops of glacial acetic acid. The reaction mixture was heated at 85–90° for 3 hours. The reaction mixture changed through a green to a brown color, a solid separated, and sulfur dioxide and carbon dioxide were evolved. The reaction product was found to be about as active as that obtained by the action of sulfuric acid on cholesterol.

The action of acetic anhydride followed by sulfoacetic acid was studied. A mixture of 0.001 mole of dry cholesterol, 4 cc. of glacial acetic acid, and 0.0025 mole of acetic anhydride was heated at 85–90° for 3 hours. Then a sample of sulfoacetic acid prepared from 0.002 mole of sulfuric acid and 0.005 mole of acetic anhydride was added and the reaction mixture was heated at 85–90° for 3 hours, during which time the colorless solution changed through a green to a brownish purple color, sulfur dioxide and carbon dioxide were evolved, and a resin separated on the sides of the flask. The product was found to be slightly more active than that produced from cholesterol by the action of the sulfuric acid-acetic anhydride reagent.

The action of fuming sulfuric acid and of chlorosulfonic acid was investigated. In a 50 cc. flask a mixture of 0.001 mole of dry cholesterol, 4 cc. of glacial acetic acid, and 0.186 gm. (equivalent to 0.002 mole of 100 per cent sulfuric acid) of 25 per cent fuming sulfuric acid was heated at 85–90° for 3 hours and the product was found to be active. Likewise, a mixture of 0.001 mole of dry cholesterol, 6.25 cc. of glacial acetic acid, and 0.002 mole (0.13 cc.) of chlorosulfonic acid (about 10 per cent excess over that amount which should be hydrolyzed by the water present in the acetic acid) was heated at 85–90° for 3 hours and the product was found to be active. Both of these reaction products possessed about the same activity as that produced by the sulfuric acid-acetic anhydride reagent.

Irradiation of Product

To determine whether a provitamin D as well as an antirachitic substance is produced by the action of sulfuric acid-acetic anhydride on cholesterol, the potency of the reaction product was compared with the potency following irradiation. In a 50 cc. flask a mixture of 0.001 mole of dry cholesterol, 4 cc. of glacial acetic acid, 0.0025 mole of acetic anhydride, and 0.002 mole of sulfuric acid was heated at 85–90° for 3 hours and the reaction product was concentrated. The residue was dissolved in 75 cc. of anhydrous ether to which 5 cc. of absolute alcohol had been added and one-half of the solution was irradiated in a 200 cc. quartz Erlenmeyer flask provided with a reflux condenser for 30 minutes at the distance of 1 foot from a Cooper Hewitt mercury arc lamp. The solution was slowly heated by means of a hot-plate to effect a more uniform irradiation. Aliquot portions of the greenish amber solution of the non-irradiated and the amber solution of the irradiated products equivalent to 6 mg. of the original cholesterol were tested biologically. Both products possessed about the same activity, which indicated that no provitamin D activatable by ultraviolet irradiation was produced by the action of sulfuric acid-acetic anhydride on cholesterol.

Heated Purified Cholesterol

The comparative efficacies of ultraviolet irradiation and sulfuric acid-acetic anhydride in imparting antirachitic activity to purified cholesterol (3) and to heated purified cholesterol were studied. The heated purified cholesterol was prepared by heating 1 gm. of purified cholesterol from room temperature to 210° during a period of 8 minutes and then heating at 210° for 2 hours in a 1 × 8 inch Pyrex tube stoppered with cotton. The oxygen of the air was not removed from the tube since Hathaway and Koch (13) found the presence of oxygen to be essential. The sublimate on the sides of the tube and the unsublimed residue were combined as it was found by Koch, Koch, and Ragins (14) that both the sublimate and the residue acquire the same degree of antirachitic potency following irradiation.

The purified cholesterol and the heated purified cholesterol were irradiated in a manner similar to the irradiation procedure de-

scribed above. A solution of 0.4 gm. of each cholesterol dissolved in 50 cc. of anhydrous ether was irradiated for 30 minutes. An aliquot of each solution was tested biologically for antirachitic activity and about 5 times as much irradiated purified cholesterol were required to produce the same degree of healing as irradiated, heated purified cholesterol. This showed that the provitamin D of heated purified cholesterol had been produced, although an accurate assay was not made, since the provitamin D was not of primary importance in this work.

The purified cholesterol and the heated purified cholesterol were treated under the same conditions with sulfuric acid-acetic anhydride. In a 50 cc. flask a mixture of 0.001 mole of the cholesterol, 4 cc. of glacial acetic acid, 0.0025 mole of acetic anhydride, and 0.002 mole of concentrated sulfuric acid was heated at 85–90° for 3 hours. After the reaction products were concentrated, an aliquot of each residue was tested biologically for activity. It was found that both reaction products gave the same degree of calcification, *i.e.* an average 2.5 plus value at the 6 mg. level. These results indicate that the provitamin D of heated purified cholesterol is not the precursor of the antirachitic substance produced by treating cholesterol with sulfuric acid-acetic anhydride.

DISCUSSION

The activation of cholesterol with the sulfuric acid-acetic anhydride reagent was investigated. It was found that to obtain the maximum potency the proportions of 0.0025 mole of acetic anhydride (98 per cent) and 0.002 mole of concentrated sulfuric acid (95 per cent) per 0.001 mole of dry cholesterol dissolved in 4 cc. of glacial acetic acid (99.5 per cent) were required. The length of time required to obtain the maximum potency, when the reaction mixture was heated at 85–90°, was found to be about 3 hours; continued heating of the reaction mixture did not influence the potency of the reaction product.

It was observed that heat was essential in the production of the antirachitic substance. When sulfuric acid and acetic anhydride are mixed below 0°, they form acetylsulfuric acid (4) which is a sulfonating reagent (8, 9). However, if acetylsulfuric acid is warmed, it is converted into sulfoacetic acid ($\text{HO}_2\text{SCH}_2\text{COOH}$), which has been used as a condensing and an

acetylating reagent (5, 6, 15). It was found that sulfoacetic acid was effective in converting cholesterol into an active product.

Although concentrated sulfuric acid converts aromatic compounds into sulfonic acids, it yields sulfate esters from alcohols and olefins. Fuming sulfuric acid and chlorosulfonic acid are sulfonating reagents (11). It was found that sulfuric acid, fuming sulfuric acid, and chlorosulfonic acid were effective in producing active products from cholesterol. Since fuming sulfuric acid reacts with acetic acid to produce sulfoacetic acid, the quantity of fuming sulfuric acid sufficient to react with about one-half of the water in the acetic acid was used in a comparison with concentrated sulfuric acid. Furthermore, it was found that, if the solution of cholesterol in acetic acid was first heated with acetic anhydride, the potency of the product obtained by the action of sulfuric acid or sulfoacetic acid was increased. The study of the various reagents indicated that the best activity was obtained when the reaction mixture was anhydrous.

Sulfur dioxide was found to be evolved when cholesterol was treated with sulfuric acid-acetic anhydride in acetic acid solution at room temperature and sulfur dioxide and carbon dioxide were evolved when cholesterol was heated in acetic acid solution with sulfuric acid, sulfoacetic acid, or sulfuric acid-acetic anhydride. The evolution of sulfur dioxide indicates that an oxidation occurs in the reaction mixture although it does not necessarily indicate that an oxidation is involved in the conversion of cholesterol into the antirachitic substance. No correlation was obtained between the potency of the various products and the quantity of sulfur dioxide evolved. This indicates that sulfur dioxide is evolved in a side reaction and not during the conversion of cholesterol into the antirachitic substance. The carbon dioxide could arise as a result of an oxidation or from a decomposition of sulfoacetic acid to form methanesulfonic acid, since carbon dioxide is evolved when the reagents are heated in acetic acid solution.

Ultraviolet irradiation of the reaction product obtained by the action of sulfuric acid-acetic anhydride on cholesterol did not affect the potency, indicating that a provitamin D was not produced. It was found that the reaction products obtained by the action of sulfuric acid-acetic anhydride on purified cholesterol and heated purified cholesterol, possessed about the same potency, demon-

strating that the provitamin D of heated purified cholesterol is not the precursor of the active substance.

SUMMARY

Cholesterol can be treated to acquire antirachitic properties by heating with sulfuric acid, sulfoacetic acid, fuming sulfuric acid, or chlorosulfonic acid in acetic acid solution. In addition to the active substance produced from cholesterol by sulfuric acid, sulfoacetic acid, or sulfuric acid-acetic anhydride, sulfur dioxide is evolved in a side reaction.

The treatment of cholesterol with sulfuric acid-acetic anhydride produces an antirachitic substance but not a provitamin D which is activatable by ultraviolet irradiation. The provitamin D of heated purified cholesterol is not the precursor of the active substance obtained by the action of sulfuric acid-acetic anhydride on cholesterol.

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THE CHEMICAL ACTIVATION OF STEROLS

IV. THE CHEMICAL ACTIVATION OF CHOLESTEROL AND CHOLESTERILENE BY VARIOUS REAGENTS*

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Cholesterol can be converted into an antirachitic product by appropriate treatment with floridin (1), sulfur trioxide, sulfuric acid-acetic anhydride (2), sulfuric acid, sulfoacetic acid, fuming sulfuric acid, and chlorosulfonic acid (3). In the process of the chemical antirachitic activation of cholesterol with these reagents, it appeared possible that the activating reagent was effective in activating cholesterol because of its dehydrating acidic properties. If the reaction is obtained because of the dehydrating acidic nature of the activating reagent, other dehydrating acidic reagents could possibly also activate cholesterol. Upon investigation, it developed that various acids and salts were effective reagents in converting cholesterol and cholesterolene into products possessing definite antirachitic activity.

Cholesteryl Sulfates

It seemed desirable to investigate the effect of heat on the potassium and ammonium salts of cholesteryl acid sulfate. Potassium cholesteryl sulfate, m. p. 288° with decomposition, and ammonium cholesteryl sulfate, m. p. 181° with decomposition, were prepared by the method of Mandel and Neuberg (4). Unnecessary heating during the preparation of these salts was avoided and they were purified by ether extraction in a continuous extractor. In agreement with the observation of Natelson and Sobel (5) that potassium cholesteryl sulfate is inactive, it was found that both of

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TABLE I

Effect of Various Chemical Reagents in Imparting Antirachitic Activity to Cholesterol and Cholesterilene

Test No.	Reaction products tested	Quantity of product fed per rat	Line test* (average plus values)	No. of rats
		mg.		
1	Potassium cholesteryl sulfate†	150	0	6
2	Ammonium " " †	150	0	6
3	Potassium " " heated	75	2 5	6
4	Cholesterilene-KHSO ₄ (1:3)	75	2.5	6
5	" (1:0.37)	75	1.5	5
6	Ammonium cholesteryl sulfate, heated	75	2.5	6
7	Cholesterilene-NH ₄ HSO ₄	75	2	6
8	Cholesterol-KHSO ₄ (250°)	75	2 5	6
9	" (215°) (1:3)	200	1.5	4
10	" (215°) (1:1)	200	1	4
11	Cholesterol-CuSO ₄	300	2.5	4
12	Cholesterol-ZnCl ₂	150	2	6
13	Cholesterol-AlCl ₃	300	1.5	5
14	Cholesterol-AlCl ₃ ·6H ₂ O	300	2	6
15	Cholesterol-HCl	150	0	6
16	Cholesterilene-HCl	150	2	6
17	Cholesterol-H ₃ PO ₄ ·Ac ₂ O	200	2	6
18	Cholesterol-H ₃ PO ₄ (HOAc)	200	0	6
19	Cholesterol-P ₂ O ₅	200	2	6
20	Cholesterilene-P ₂ O ₅	200	3.5	6
21	Dicholesteryl acid phosphate, heated	300	2 5	5
22	Cholesterilene-H ₃ PO ₄	300	0‡	6
23	Cholesterol-H ₃ PO ₄	300	0	6
24	KH ₂ PO ₄	110	0‡	6
25	Cholesterol-CCl ₃ COOH (HOAc)	150	0	6
26	" (60°)	150	0	5
27	" (100°)	150	1.5	6
28	" (160°)	150	2.5	5
29	Cholesterol-soda lime	300	0	4

* A 2 plus value here denotes approximately 5 vitamin D units, United States Pharmacopoeia XI (6).

† The cholesteryl sulfate salts were powdered and intimately mixed with ball-milled rachitogenic Ration 2965 (7). All other reaction products were dissolved in a solvent and the solution evaporated on the rachitogenic ration.

‡ One of the six rats showed a speck of calcification.

these salts were inactive when tested biologically. The results of these tests (Nos. 1 and 2) and all others referred to hereinafter are presented in Table I.

The effect of heat on the cholesteryl sulfate salts was investigated. In a 1 × 8 inch Pyrex tube stoppered with cotton, 2 gm. of potassium cholesteryl sulfate were heated at 250° for 30 minutes in a preheated Wood's metal bath. After cooling, the brown product was extracted with anhydrous ether. The ether-insoluble material was found to be potassium acid sulfate and the ether extract was found to possess antirachitic activity (Test 3). The cleavage of potassium acid sulfate from the potassium cholesteryl sulfate could have resulted in the formation of a new carbon-carbon double bond and it is possible that the active substance could have been produced at the time of cleavage or that a cholestadiene was formed which was then converted into an antirachitic substance. When 1 gm. of dry cholesterilene¹ was heated with 3 gm. of powdered, freshly fused potassium acid sulfate at 250° for 30 minutes, the ether extract of the brown reaction product was found to be active (Test 4). However, the product obtained by heating 1 gm. of dry cholesterilene with 0.37 gm. of potassium acid sulfate (1 mole per mole) at 250° for 30 minutes was found to be slightly less active (Test 5).

When 2 gm. of ammonium cholesteryl sulfate were heated at 220° for 30 minutes, it yielded ammonium acid sulfate and an active ether extract (Test 6). The ether extract of the reaction product obtained by heating 1 gm. of dry cholesterilene with 3 gm. of ammonium acid sulfate at 220° for 30 minutes was likewise found to be active (Test 7).

Various Salts

The action of various salts was studied to determine whether they were effective reagents in converting cholesterol into an antirachitic product. Since potassium acid sulfate is a dehydrating agent, it appeared that it should activate cholesterol as well as cholesterilene. The ether extract of the brown reaction product obtained by heating a mixture of 1 gm. of dry cholesterol and 3

¹ The cholesterilene, m. p. 78-79°, used in the experiments described in this paper, was prepared from Wilson's C.P. cholesterol by the copper sulfate method of Mauthner and Suida (8).

gm. of powdered, freshly fused potassium acid sulfate at 250° for 30 minutes was found to be active (Test 8). However, the proportion of the potassium acid sulfate as well as the temperature was found to be important. For example, the product obtained by heating 1 gm. of cholesterol with 3 gm. of potassium acid sulfate at 215° for 20 minutes was found to possess less activity (Test 9) and the product obtained by heating 1 gm. of cholesterol with 1 gm. of potassium acid sulfate at 215° for 20 minutes was found to be even less active (Test 10). Cholesterilene and dicholesteryl ether were obtained by heating 1 gm. of cholesterol with 1 gm. of potassium acid sulfate at 215° for 20 minutes, whereas the product obtained by heating 1 gm. of cholesterol with 3 gm. of potassium acid sulfate at 215° for 20 minutes was difficult to handle.

The ether extract of the cooled brown reaction product obtained by heating an intimate mixture of 2 gm. of dry cholesterol and 6 gm. of anhydrous copper sulfate at 250° for 30 minutes was found to be active (Test 11). The ether-insoluble material, although not completely soluble in water, gave a green aqueous solution similar to the color of an acid copper sulfate solution and a small copper-colored mirror was observed on the bottom of the tube. This indicated that the copper sulfate had decomposed. Other preparations made by heating cholesterol with copper sulfate under various conditions yielded products which gave a positive test for organic sulfur and which rendered the rations supplemented with these products unpalatable to rats.

A mixture of 1 gm. of dry cholesterol and 3 gm. of anhydrous zinc chloride (Kahlbaum) was heated at 225° for 15 minutes. After cooling, distilled water was added and the yellow reaction product was extracted with ether. The ether extract was washed with sodium carbonate solution, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The ether extract residue was found to be active (Test 12).

In a 200 cc. 3-necked round bottomed flask provided with a mechanical stirrer and a reflux condenser to which was attached a calcium chloride tube were placed 2 gm. of dry cholesterol, 50 cc. of dry benzene, and 3 gm. of anhydrous aluminum chloride. The reaction mixture was mildly refluxed for 8 minutes during which time a red color developed. The reaction product was

then hydrolyzed with water and the yellow benzene layer was washed with water, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was found to possess antirachitic properties (Test 13).

A mixture of 2 gm. of cholesterol and 6 gm. of aluminum chloride hexahydrate was heated from room temperature to 180° during a period of 8 minutes and then heated at 180° for 45 minutes. After cooling, the yellow reaction product was washed with distilled water and extracted with ether. The ether extract was washed with sodium carbonate solution, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The pale yellow ether extract residue was found to be active (Test 14).

Hydrogen Chloride

In connection with the action of the chlorides just described, it seemed desirable to determine whether hydrogen chloride would convert cholesterol or cholesterolene into an antirachitic product. In a pressure bottle, a solution of 1 gm. of dry cholesterol dissolved in 30 cc. of anhydrous ether was saturated with dry hydrogen chloride at 0° during a period of 3 hours and then allowed to stand stoppered at room temperature for 3 days. Crystals separated from the colorless solution during the addition of the hydrogen chloride but had redissolved in 24 hours. After cooling, the bottle was opened and the colorless solution was concentrated *in vacuo*. The white solid residue was found to be antirachitically inactive (Test 15).

1 gm. of dry cholesterolene was treated likewise with hydrogen chloride. During the addition of the hydrogen chloride, the solution changed through a yellow, to a light brown, to a reddish brown color with a green fluorescence. The light brown reaction product residue was found to possess antirachitic properties (Test 16).

Phosphoric Acid and Anhydride

In analogy with the action of sulfuric acid-acetic anhydride, cholesterol was treated with phosphoric acid-acetic anhydride in the molar proportions of 2 moles of phosphoric acid and 2.5 moles of acetic anhydride per mole of cholesterol. In a 50 cc. Erlenmeyer flask a mixture of 1 gm. of dry cholesterol, 0.51 gm.

of crystalline orthophosphoric acid, 0.61 cc. of acetic anhydride, and 10 cc. of glacial acetic acid was heated at 85–90° for 3 hours. The reaction product was concentrated and the brown residue was found to be active (Test 17). The action of phosphoric acid in the ratio of 2 moles per mole of cholesterol was also investigated. The pale yellow reaction product obtained by heating 1 gm. of cholesterol with 0.51 gm. of crystalline orthophosphoric acid in 10 cc. of glacial acetic acid at 85–90° for 3 hours was found to be inactive (Test 18).

The effect of heating a mixture of molar equivalents of cholesterol and phosphoric anhydride was investigated. In a 1 × 8 inch Pyrex tube a mixture of 1.087 gm. of dry cholesterol and 0.4 gm. of phosphoric anhydride was heated at 210° for 15 minutes; the phosphoric anhydride was introduced into the tube on a small watch-glass. The reaction mixture changed through a purple to a brown color and the reaction product was found to be active (Test 19). Since phosphoric anhydride is a dehydrating agent, it was desirable to determine whether the water formed by the dehydration of the cholesterol had any effect. It was found that when a mixture of 1.037 gm. of dry cholesterilene and 0.4 gm. of phosphoric anhydride was heated at 210° for 15 minutes, the brown reaction product was found to be more active (Test 20) than the product obtained by the action of the phosphoric anhydride on cholesterol.

When dicholesteryl acid phosphate is heated for a few seconds at 200°, it yields cholesterilene and probably orthophosphoric acid (9). In a tube, 2 gm. of dicholesteryl acid phosphate, m.p. 204° with decomposition, were heated at 225° for 10 minutes and the brown reaction product was found to be active (Test 21). However, the same result was not obtained when cholesterilene or cholesterol was heated under the same conditions with orthophosphoric acid in the ratio of 2 moles per mole. The reaction products obtained by heating 2.028 gm. of dry cholesterilene or 2.127 gm. of cholesterol with 0.27 gm. of crystalline orthophosphoric acid at 225° for 10 minutes were found to be inactive (Tests 22 and 23).

A control ration was compounded to provide phosphorus as monopotassium phosphate, in a quantity larger than the amounts in the various phosphorus-containing samples and was fed simul-

taneously to determine whether the calcification was due to the antirachitic product or to changing the calcium to phosphorus ratio by the addition of phosphorus to the rachitogenic ration. No detectable healing calcification was evident in the rachitic metaphyses of rats fed these control rations (Test 24).

Trichloroacetic Acid

It seemed desirable to determine whether a highly ionizable organic acid would convert cholesterol into an antirachitic product, so the action of trichloroacetic acid was investigated. In a 50 cc. Erlenmeyer flask, 1 gm. of dry cholesterol was heated with 0.85 gm. of freshly distilled trichloroacetic acid dissolved in 4 cc. of glacial acetic acid at 85–90° for 3 hours and the colorless reaction product was concentrated *in vacuo*. After treatment with distilled water, the yellow ether-soluble residue was found to be inactive (Test 25).

In a tube, 1 gm. of dry cholesterol was heated with 3 gm. of trichloroacetic acid at 60° for 1 hour during which time the reaction mixture became red in color. After treatment with distilled water, the pale yellow ether-soluble residue was found to be inactive (Test 26). The reaction was repeated by heating the reaction mixture at 100° for 1 hour and the dark red reaction product changed to a light brown color on being washed with distilled water and was found to be slightly active (Test 27). The reaction was also repeated by heating the reaction mixture at 160° for 10 minutes during which time a color change of purple to brown was observed. After treatment with distilled water, the brown solid product was found to be the most active (Test 28) produced with this reagent under the various conditions which were used. The wash water gave a positive test for chloride ion, whereas an aqueous solution of the trichloroacetic acid gave only a very slight turbidity with silver nitrate solution. Therefore, it is possible that the trichloroacetic acid decomposed somewhat during the reaction.

Soda Lime

In a tube, 2 gm. of dry cholesterol were heated with 2 gm. of soda lime at 210° for 30 minutes. The light brown reaction product was washed with water and extracted with ether. The yellow

ether extract was washed with water, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was found to be inactive (Test 29).

DISCUSSION

Potassium and ammonium cholesteryl sulfates were found to decompose on heating above their melting points to yield antirachitic products and potassium or ammonium acid sulfate respectively. Dicholesteryl acid phosphate was also found to decompose above its melting point to yield an antirachitic product. It is possible that the active substance could have been formed at the time of cleavage or could have resulted from a reaction between the substances formed by the cleavage. The reaction products obtained by heating cholesterilene with potassium acid sulfate or orthophosphoric acid were less active than the reaction products obtained by heating potassium cholesteryl sulfate or dicholesteryl acid phosphate under the same respective conditions. This would indicate that more is involved in the production of the antirachitic product than the formation of cholesterilene and then the conversion of this to an active substance by the inorganic reagent formed by the cleavage.

Potassium acid sulfate was found to be effective in converting cholesterol as well as cholesterilene into an antirachitic product. It was observed that the temperature and the proportion of the potassium acid sulfate were important in influencing the potency of the reaction product. Under mild conditions dehydration to cholesterilene and dicholesteryl ether is favored, whereas the reaction products obtained by heating at higher temperatures possess definite antirachitic activity. Montignie (10) has shown that dicholesteryl ether is produced by heating cholesterol with potassium acid sulfate in the ratio of 1 gm. per 4 to 5 gm. at 145° for 30 minutes. The reaction product obtained by heating cholesterol with potassium acid sulfate at 220° in the ratio of 1 gm. per 3 gm. was found to be more active than that obtained by heating the mixture in the ratio of 1 gm. per gm.; under the latter conditions, cholesterilene and dicholesteryl ether can be conveniently prepared. Dehydration also seems to be important in the production of an antirachitic substance with hydrogen chloride, since an antirachitic product was not obtained by treating chole-

terol with hydrogen chloride under the same conditions which converted cholesterol into an active product.

Koch, Koch, and Ragins (11) found that heat alone or heat in the presence of soda lime produces a provitamin D in purified cholesterol, whereas heating with aluminum chloride, phosphoric anhydride, or copper sulfate did not. It is interesting to note that these three reagents which did not produce a provitamin D were found to convert cholesterol into an antirachitic product, whereas an antirachitic product was not obtained with soda lime. Cholesterol was also found to be converted into an active reaction product by heating with either anhydrous zinc chloride or aluminum chloride hexahydrate. The reaction product obtained by heating cholesterol with aluminum chloride in benzene solution was found to be active, although an antirachitic product was not obtained by heating a mixture of cholesterol and aluminum chloride in the absence of a solvent.

Phosphoric acid-acetic anhydride was found to be similar to the sulfuric acid-acetic anhydride reagent in converting cholesterol into an active product, except that the former reagent was less efficient in producing an active substance. Phosphoric acid in the absence of acetic anhydride was found ineffective in converting cholesterol into an antirachitic product, whereas sulfuric acid yielded an active product when used under the same conditions (3). The reaction product obtained by heating dry cholesterol with phosphoric anhydride was found to be more active than that produced from cholesterol.

From the results obtained with trichloroacetic acid, it appears that the active substance produced does not result merely because trichloroacetic acid is a highly ionizable acid. It may be that dehydration occurs, since, although cholesterol does not give the Rosenheim reaction in the cold, it does give the characteristic red color when heated and it has been pointed out (12) that the Rosenheim reaction is positive for sterols possessing a conjugated double bond system or by those capable of forming such a system by the action of trichloroacetic acid which, in all probability, is dehydrating. In addition to dehydration, the decomposition of the trichloroacetic acid probably is involved in the process of the conversion of cholesterol into an antirachitic product.

SUMMARY

It was found that various acids and salts are effective reagents in converting cholesterol and cholesterilene into antirachitic products. The temperature, time, and the proportion of the reagent were found to be important.

Potassium and ammonium cholesteryl sulfates decompose on heating above their melting points to form antirachitic products and the respective potassium or ammonium acid sulfate. Dicholesteryl acid phosphate also decomposes on heating above its melting point to yield an antirachitic product.

Cholesterol can be converted into an antirachitic product by heating with potassium acid sulfate, copper sulfate, zinc chloride, aluminum chloride hexahydrate, phosphoric anhydride, or trichloroacetic acid. Cholesterol also yields an antirachitic product when heated with aluminum chloride in benzene solution or when heated with phosphoric acid-acetic anhydride in acetic acid solution.

Cholesterilene yields an antirachitic product when heated with potassium acid sulfate, ammonium acid sulfate, or phosphoric anhydride. Cholesterilene can also be converted into an antirachitic product when treated with hydrogen chloride in ether solution.

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THE WAX-LIKE CONSTITUENTS OF THE CUTICLE OF THE CHERRY, *PRUNUS AVIUM*, L.*

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In previous papers we have recorded the results of investigations dealing with the isolation and identification of the wax-like surface constituents of apples (1), pears (2), and cranberries (3). In continuation of the research on cuticle waxes the results of a study of similar substances from the cherry are herewith presented.

A survey of the literature (4) indicates that although considerable work of a chemical nature has been reported on various organs of the sweet cherry, *Prunus avium*, L., the cuticle wax of the fruit seems to have been entirely neglected.

For the purpose of the present study 49.9 kilos of fresh Bing cherries, presumably grown in the Pacific Northwest, were purchased from the Washington, D. C., market. After the cherries were pitted and pressed to remove as much juice and pulp as possible, the press-cake was washed with water and again pressed. The pressed skins were dried first in a current of slightly warm air, then over steam pipes, and finally in a desiccator over calcium chloride. The ground, dehydrated skins (1.2 kilos) were exhaustively extracted, first with petroleum ether and then with ethyl ether. The total petroleum ether extract amounted to 9.8 gm. and the ether extract to 1.6 gm., corresponding to 0.8 and 0.1 per cent respectively of the dried skins.

Petroleum Ether Extract—The yellowish green petroleum ether extract, which had a free acidity equivalent to 22.2 per cent calculated as oleic acid, was saponified with alcoholic potassium hydroxide and the unsaponifiable matter separated from the soap solution by extraction with ether. The yield of unsaponifiable

* Food Research Division Contribution, No. 329.

material amounted to 2 gm. and the acids to nearly 7 gm. The remaining aqueous filtrate, after removal of the acids, was examined for the presence of glycerol. A small amount of a colorless viscous liquid was obtained which responded to all the usual tests for glycerol.

Fatty Acids—The fatty acids derived from the soluble soaps after acidification and extraction with ether had an iodine number of 72 (Rosenmund-Kuhnhehn method (5)). The solid and liquid acids were separated by the lead salt-ether method into 2 gm. of solid and 2 gm. of liquid acids.

Palmitic and Stearic Acids—The solid acids were esterified with 5 per cent hydrochloric acid in ethanol and, since fractionation yielded no single pure ester, the acids were regenerated and crystallized from ethanol-ethyl acetate solution. The main fraction melted at 53.0–53.3° and set solid at 51.5°. The neutralization value was found to be 201.6 and the molecular weight 278.3. Analysis gave C 75.99, H 12.33.¹ The melting point of the material suggested a 70 to 30 binary mixture of palmitic and stearic acids (6, 7); however, the mean molecular weight and the carbon and hydrogen values were much too high for this mixture and indicated the presence of an acid higher than C₁₈. Since such mixtures are difficult to separate, especially so with the limited material available, it must suffice to say that the saturated acid fraction consisted of at least a ternary mixture of palmitic, stearic, and a small amount of acid higher than C₁₈.

Linoleic and Oleic Acids—Examination of the liquid acid fraction showed that it contained only linoleic and oleic acids. The composition of the mixture was therefore computed from the iodine value (105.9, Rosenmund-Kuhnhehn method), which indicated the presence of 17.7 per cent of linoleic acid and 82.5 per cent oleic acid.

The remaining liquid acid fraction was dissolved in ether and brominated dropwise in a bath maintained at –13°. No hexabromide could be separated from the brominated mixture; consequently excess of bromine was destroyed with β -amylenes and the ether removed by evaporation. The residue was extracted with

¹ The writers are greatly indebted to Mrs. Mildred S. Sherman, Fertilizer Investigations, Bureau of Chemistry and Soils, for the analytical results reported.

petroleum ether and the extract permitted to stand at a low temperature until the tetrabromide had separated. After separation and recrystallization, the tetrabromide was found to melt at 114.0–114.5° and gave on analysis C 35.31, H 5.38, Br 54.99. Theory for tetrabromolinoleic acid, $C_{18}H_{32}O_2Br_4$, requires C 36.00, H 5.38, Br 53.28.

The residual solution after removal of the tetrabromide fraction was evaporated and the dibromostearic acid debrominated with zinc dust in alcoholic solution. The recovered oleic acid had an iodine value of 90.8 (Rosenmund-Kühn method). Theory for oleic acid is 89.93.

Paraffin Hydrocarbons—The small quantity of unsaponifiable material (2 gm.) was not sufficient for the isolation of oxygenated compounds and it was therefore submitted to treatment with concentrated sulfuric acid at 110°. When further treatment with a fresh quantity of acid no longer gave any indication of darkening, the remaining product was recovered and crystallized from petroleum ether-acetone solution. The material consisted of plates melting at 63.2–63.5° and setting to a crystalline solid at 62.7–62.5°. Although the melting and setting points were quite sharp, the transition points were much depressed and obscure, indicating that the hydrocarbon fraction was a mixture. The x-ray spacing² obtained with Cu K_α radiation was found to be 39.00 ± 0.20 Å. Comparison of these values with the figures obtained by Piper, Chibnall, and coworkers (8) on pure synthetic hydrocarbons and their mixtures indicates that the cherry hydrocarbon fraction consists predominantly of nonacosane, $C_{29}H_{60}$, admixed with a hydrocarbon of longer chain length.

Ether Extract—The residue from the ether extract of the cherry skins which had been previously extracted with petroleum ether was dissolved in dilute ethanol containing an excess of sodium hydroxide. After filtering, the ethanol was gradually evaporated, whereupon two main fractions were obtained; one was less soluble (A), while the other fraction (B) was much more soluble and did not separate until most of the ethanol was removed.

d-Glucosidylsterol—The less soluble fraction (A) was washed with ether, in which it was now only slightly soluble, then recryst-

² Measurements of the crystal spacings were kindly made by Dr. Sterling B. Hendricks, Fertilizer Investigations, Bureau of Chemistry and Soils.

tallized successively from amyl acetate, ethanol, glacial acetic acid to which a little water was added, and finally from pyridine. Obtained in this manner, it melted at 265–266° and gave positive Liebermann-Buchard and Molisch tests. Since the above data indicated the possibility of the substance being a phytosterolin, it was converted into its acetyl derivative by boiling with acetic anhydride and anhydrous sodium acetate. Recrystallized from dilute ethanol, the derivative melted at 164.5–165°. Analysis of the acetate gave C 69.36, H 9.30. *d*-Glucosidylsitosterol tetraacetate, $C_{42}H_{78}O_{10}$, melts at 166.3–167° (9) and requires C 69.29, H 9.20.

Ursolic Acid—The more soluble fraction (B), which from its behavior appeared to be sodium ursolate, was redissolved in ethanol containing a slight excess of sodium hydroxide and an equal quantity of hot water was added. The crystalline salt separated after evaporation of most of the ethanol by boiling. The parent substance was regenerated by solution of the salt in ethanol and addition of dilute hydrochloric acid. The recovered acid was finally recrystallized from absolute ethanol, from which it separated as needles melting at 281–282°. Analysis gave C 78.74, H 10.67. Ursolic acid, $C_{30}H_{48}O_3$, requires C 78.88, H 10.60.

The methyl derivative prepared with diazomethane was recrystallized from dilute ethanol, after which it melted at 168.2–168.5°. Analysis gave C 78.98, H 10.93, OCH_3 5.93. Monomethyl ursolate, $HO \cdot C_{29}H_{46} \cdot COO(CH_3)$ requires C 79.08, H 10.71, OCH_3 6.60.

SUMMARY

The skins of Bing cherries (*Prunus avium*, L.) have been examined with respect to the constituents soluble in petroleum ether and ethyl ether. From the petroleum ether extract there have been isolated or identified solid fatty acids consisting of a ternary mixture of *palmitic*, *stearic*, and a small amount of acid higher than C_{18} ; liquid fatty acids, *linoleic* and *oleic acids*; a small amount of *glycerol*; and a hydrocarbon fraction consisting predominantly of *nonacosane*, admixed with a hydrocarbon of longer chain length. The ether extracted yielded *d*-glucosidylsitosterol and *ursolic acid*.

The yields of the petroleum ether and ethyl ether extracts amounted to 0.8 per cent and 0.1 per cent, respectively, of the dried skins. A comparison of these figures with the corresponding percentage yields from apple and pear cuticles (2) indicates that herein may be the explanation for the less efficient protective surface coating of the cherry.

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THE FRUCTOSE CONTENT OF SPINAL FLUID

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In 1935 the presence of a compound having the properties of fructose was noted during the study of a specimen of spinal fluid taken from a patient with encephalitis (1). Since an application of similar methods to blood had failed to demonstrate the presence of significant amounts of such a substance (2) and since it is believed that the sugars of spinal fluid are derived from those in the blood (3), an investigation of a number of fluids sent to the laboratory for routine study was undertaken. In a preliminary note (4) it was reported that fructose was apparently present in most specimens of spinal fluid, that the amount seemed to parallel the concentration of total sugar which the specimens contained, but that glucose itself gave a reaction with the reagents used in the fructose determination. Consideration of these facts made it evident that it would be necessary to determine both glucosé and fructose simultaneously in spinal fluid, and to try to make some correction for the reaction given by the first named sugar. As glucose values of spinal fluid frequently change upon standing (5), this meant that analyses would be satisfactory only if they were carried out upon freshly obtained fluid. Since this precaution had not been taken in the first series, a new investigation was undertaken. Soon after the technique for such an investigation had been worked out, an opportunity for studying two cases of epidemic meningitis presented itself. The result of the study of these two cases has already been described (6). Fructose was found in all specimens examined in concentrations which approximately paralleled the amount of glucose which was present. By incubation of the fluids it was possible to destroy the glucose almost completely without measurably affecting the fructose. Since such a destruction was not observed in specimens from which

cells had been removed by centrifuging, and since an addition of the cells separated from meningitic fluid caused a similar differential destruction of sugar in normal fluid, the reaction appeared to be due to the action of a cellular enzyme.

The present paper contains results of similar analyses of fluids of various types. The material serves several purposes. Results of studies of normal, or practically normal, material provide a necessary contrast with those which were carried out upon abnormal fluids from meningitic patients; blood and spinal fluid specimens were collected simultaneously to facilitate the study of possible explanations of the presence of fructose in the fluid; and, lastly, a series of control experiments, which confirmed and amplified results presented in the previous publications, was carried out and will be discussed.

In the preparation of specimens of blood and spinal fluid for analysis protein and other interfering compounds were precipitated at once with zinc sulfate and sodium hydroxide, as recommended by Somogyi (7). The blood was so treated as to give a 1:10 dilution, but spinal fluid, in which little protein is present, was diluted much less (usually less than 1:2) to provide a more concentrated filtrate for the final analyses. Zinc and sodium hydroxide were present in all instances in the proportions recommended by Somogyi, *i.e.* 1 cc. of 10 per cent zinc sulfate and 1 cc. of 0.5 N sodium hydroxide in each 10 cc. of the final mixture. Precipitated protein and zinc hydroxide were removed by centrifuging. Glucose was determined in the supernatant fluid by the method of Folin and Wu (8), modified, when the sugar content was very low, as recommended by Hubbard and Allison (9). Fructose was determined by the application of the resorcinol technique described by Roe (10). Because the concentration of fructose varied markedly in different samples, a series of standards containing concentrations of pure fructose ranging from 0.1 to 4.0 mg. per 100 cc. was regularly analyzed simultaneously with the blood and fluid filtrates. The color of the "unknown" was compared with that of the standard nearest to it in tint in a colorimeter when the concentration of fructose was greater than 0.5 mg. per 100 cc.; otherwise an approximate reading was made by comparing the color with that of the weak standards in tubes of equal diameter.

As mentioned in the preliminary note, glucose gives some color when treated with hydrochloric acid and resorcinol according to Roe's technique. This color is so slight that in the concentration in which the sugar occurs in blood filtrates it is relatively unimportant. In the more concentrated spinal fluid filtrates, however, glucose was present in much higher concentration, and it, therefore, seemed necessary to investigate this reaction carefully. Many samples of pure glucose, including one from the Bureau of Standards, were repeatedly analyzed according to Roe's procedure. All gave a color which varied in intensity with the concentration of the sugar present, and which closely resembled that given by fructose. The type of the reaction of the two sugars was decidedly different. The color from fructose reached a maximum intensity in about 10 minutes and diminished somewhat when heating was continued for 0.5 hour. The color given by glucose developed much more slowly, and, in dilute solutions did not appear to reach maximum values until the solutions had been heated for 30 minutes. These colors were relatively very faint. After a 10 minute period the color given by 20 mg. of glucose per 100 cc. was approximately equal to that obtained from 0.2 mg. of fructose in 100 cc. Even after heating for an hour the color given by 10 mg. of glucose was equivalent only to that from 0.15 mg. of fructose.

Not only were these colors different in intensity; they were also different in kind. In the preliminary note it was stated that there was a difference between the absorption spectra of the colored substances obtained from glucose and fructose. Comparisons of such spectra have been made repeatedly during the present investigation with glucose from various sources. In each instance the results have been the same; there was a general similarity between the spectra, but relative absorptions of light in the red and violet ends of the spectrum were significantly different.

Apparently the differences in tint affected somewhat the colorimetric estimation, for the readings when fructose was compared with mixtures of glucose and fructose were not exactly the ones which would have been predicted from the separate analyses of the two sugars. This made the estimation of the correction for analyses of blood and spinal fluid difficult. It was necessary to

prepare one correction table for use when fructose was absent or present only in very low concentrations, and others for use when concentrations over 0.5 mg. per 100 cc. of the sugar were present. These correction values were significantly, but not markedly, different, and both sets of values paralleled closely, but not exactly, the amount of the glucose analyzed. In practise the application of this glucose correction was based upon the total reducing power of the solutions, as determined by the method of Folin and Wu; for fructose formed only a small per cent of the total sugar present, and the amount of non-sugar reducing compounds in material clarified by the method of Somogyi is minimal.

It is evident from these facts that, if analyses seem to show that very small amounts of fructose are present in solutions containing large concentrations of glucose, the significance of the fructose value is very uncertain. In the determinations upon blood reported below the apparent concentration of fructose in the filtrate, equivalent to a 1:10 dilution of blood, was usually from 0.1 to 0.2 mg. per 100 cc. Such concentrations give very faint colors which can be measured only approximately. Furthermore, glucose solutions of the concentrations present in these filtrates gave colors which would account for two-thirds of all the color found, and the accuracy of these glucose corrections was also low. It seems to the authors that the corrected determinations had a probable error equivalent to from 1 to 2 mg. of fructose per 100 cc. of blood—figures which in most instances exceeded the amount of the sugar found.

The fructose values determined in spinal fluid seemed to be much more satisfactory. The amount of color produced in the filtrate was great enough to be read in a colorimeter, and the glucose correction, while significant, accounted on the average for only 15 per cent of all the color found. In a number of experiments determinations were carried out upon different aliquots of the same spinal fluid. The results of such analyses agreed well, and showed that the accuracy of the analysis was about 5 per cent when much fructose was present, and 0.1 to 0.2 mg. per 100 cc. when the concentration of the sugar was low.

One of the points emphasized in the previous papers, and confirmed by the results to be described below, was the marked difference between the apparent fructose content of blood and spinal

fluid. It seemed desirable to try to exclude possible technical causes of this finding. The authors thought that it might be explained by the marked difference in the protein content of the two fluids; *i.e.*, that the protein precipitate might remove from solution significant amounts of the substance giving the resorcinol reaction. To test this hypothesis they prepared mixtures of spinal fluid with blood, with plasma, and with plasma rendered sugar-free by incubation with massive suspensions of yeast cells. These mixtures were precipitated by Somogyi's method and analyzed for fructose. The results obtained agreed closely with those predicted from separate analyses of the components of the mixtures. No evidence that the active substance in spinal fluid was removed with the precipitate was obtained. The effect of the precipitating reagents upon the reactive substance was also investigated. In a number of instances untreated "normal" fluids were analyzed simultaneously with filtrates prepared from them by the technique described above; identical results were regularly obtained.

As part of the inquiry into the technical limitations upon the method, the effect of allowing spinal fluid specimens and filtrates prepared by Somogyi's method to stand before analyzing them was investigated. Within the limits of error given above, comparable results were obtained upon filtrates when the analysis was completed within 20 minutes of the time when the fluids were obtained and when they stood 6 hours at room temperature before the colors were developed. In our experience neither filtrates nor untreated fluid showed a significant change after 24 hours in the ice box. When unprecipitated fluid stood at higher temperatures, the results were unsatisfactory.

Experiments which were planned to determine the nature of the substance giving the reaction with resorcinol were described briefly in the preliminary note. These experiments have been repeated and others bearing upon the question have been carried out during the present investigation. Studies of the nature of the compound were first made by a biological method. Short incubation with massive suspensions of yeast cells destroys glucose, and procedures based upon this reaction have been considered quite specific and have been used in the determination of the true glucose content of blood (11). Fructose can be destroyed in the

same way (12). Short (10 to 15 minutes) incubation with massive suspensions of washed yeast cells has been used many times upon spinal fluid specimens from patients with various clinical conditions and complete removal of the reactive substance has been regularly demonstrated.

Since glucose and fructose are both destroyed by this technique, and since the filtrate prepared from spinal fluid contains relatively high concentrations of glucose, experiments were carried out to determine definitely whether two different sugars were present in the fluids. Freshly prepared and old glucose solutions were analyzed repeatedly, both separately and simultaneously with spinal fluid specimens. In no instance was enough glucose, measured by the total reducing power, present in any spinal fluid specimen to account for the intensity of the resorcinol reaction which it gave.¹ Studies of the absorption spectra obtained when spinal fluid was treated with the resorcinol reagent have been made repeatedly. These spectra were regularly different from the absorption spectrum of the colored compound given by glucose, and approximated quite closely that obtained from fructose. As further evidence that the reactive substance is not wholly glucose, the incubation experiments reported in the paper upon the two cases of meningitis may be cited; in these experiments, as already noted, the glucose was almost wholly destroyed and the fructose little affected. All of these experiments taken together make it seem certain that the substance in spinal fluid which gave the greater part of the color obtained with the resorcinol reagent was not glucose, but some compound closely resembling fructose.

The possibility of the presence of other compounds besides glucose and fructose must be considered. Sucrose gives the resorcinol reaction and is destroyed by short incubation with yeast cells (12). It is not, however, affected by certain strains of bacteria which destroy glucose and fructose (13). All of the reactive compound was removed from spinal fluid specimens which were inoculated with such organisms and incubated for 24 hours. The reactive substance, therefore, was not sucrose.

¹ In one sample from a diabetic patient the "total fructose" as measured directly by the resorcinol reaction was 11.1 mg. per 100 cc. The "total glucose," measured by the reducing power, was 200 mg. per 100 cc. Pure glucose solutions of this concentration give a color which is somewhat less than that given by 2 mg. of fructose in 100 cc.

Certain hexosephosphoric acid esters would be destroyed by these biological methods and would give a resorcinol reaction identical with that given by fructose (14). It seems improbable that the substance studied in spinal fluid belongs to this group of compounds, for there is usually not enough organic phosphorus present in spinal fluid to account for all of the apparent fructose found by us (15). As a rather extreme example, studies of a specimen of fluid from a diabetic patient may be cited. Only 1.6 mg. per 100 cc. of total phosphorus were found simultaneously with a fructose concentration of 48 mg. per 100 cc. If all of the phosphorus, inorganic as well as organic, had been in the form of hexosemonophosphate, only about one-sixth of the sugar would have been accounted for.

On the basis of these control experiments it seems proper to accept the corrected figures based upon estimations of the color produced by the resorcinol reaction as measuring fructose in spinal fluid. Detailed results of analyses carried out upon blood and spinal fluid specimens are given below.

A brief summary of the essential data upon each of the patients studied is given in Table I. It seems to the authors that a sufficiently wide range of clinical condition is included in the group to serve as a valid basis for the study. The spinal fluid of approximately two-thirds of the subjects was essentially normal. None of the studies has been reported previously.

In Table II are given the results of chemical analyses, made as described above, upon specimens of blood and spinal fluid drawn simultaneously from these patients. Table II includes the values found for "fructose" on direct measurement of the color obtained from filtrates, as well as their estimated fructose content. The difference between the two sets of values is due to the color given by glucose. The correction was determined as described.

The probable accuracy of the fructose determinations in blood and spinal fluid has already been discussed. It is evident that little significance can be attached to the individual blood values. The authors believe that traces of fructose were probably present in many of the blood specimens studied, but even such a conservative conclusion as this is not wholly justified by the figures, because the magnitude of the glucose correction under the conditions pertaining in these analyses could not be determined with cer-

TABLE I
Description of Material

Series No.	Race	Sex	Age	Diagnosis	Spinal fluid
			<i>yrs.</i>		
1	White	M.	50	Generalized arteriosclerosis; traumatic neurosis	Normal
2	"	"	34	Posttraumatic encephalopathy	"
3	"	"	21	Posthemorrhagic encephalopathy	"
4	"	"	30	Disease of brain and cord, undiagnosed	"
5	"	"	19	Tuberculosis	"
6	"	"	76	Syphilis	"
7	"	F.	34	Lymphadenitis	"
8	Negro	"	36	Fever of undetermined origin	"
9	White	M.	49	Tertiary syphilis	"
10	"	F.	67	Diabetes	"
11	"	M.	27	Syphilis; psychosis	"
12	"	F.	54	Encephalitis (secondary); cerebral thrombosis	"
13	"	"	17	Anxiety state	"
14	"	M.	36	Syphilis of central nervous system	Wassermann +; gold curve +
15	"	F.	43	" "	Same
16	"	"	25	Multiple sclerosis	Normal except gold curve +
17	"	M.	16	Convulsive state, myoclonic type	Normal
18	"	F.	30	Hysteria	"
19	"	"	40	Syphilis	"
20	"	"	49	"	"
21	"	M.	28	"	"
22	"	F.	67	Encephalopathy (due to lead?)	"
23	"	"	41	Diagnostic spinal tap (no diagnosis)	"
24	"	M.	53	Cerebral arteriosclerosis	"
25	Negro	F.	63	" hemorrhage	Protein 0.5%
26	White	M.	21	Tuberculous meningitis	Typical of condition
27	"	"	44	Epidemic meningitis	" "
28	"	"	67	" "	" "
29	"	F.	24	Tuberculous meningitis	" "
30	"	M.	29	" "	" "

TABLE II
Results of Sugar Analyses

Series No.	Date	Whole blood			Cerebrospinal fluid		
		"Glucose"	"Fructose"		"Glucose"	"Fructose"	
			Corrected	Un-corrected		Corrected	Un-corrected
	1936	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
1	Feb. 6	81	0.2	1.0	73	3.0	3.6
2	Mar. 5	93	0.6	1.5	72	3.3	3.8
3	" 5	109	0.0	1.0	63	2.3	2.7
4	" 9	140	0.6	2.0	72	3.0	3.6
5	May 20	78	0.3	1.1	73	6.7	7.3
6	" 22	79	0.2	1.0	75	2.8	3.6
7	" 26	149	1.5	3.0	62	2.3	2.6
8	" 28	88	0.6	1.5	95	2.9	3.6
9	" 28	76	0.2	1.0	37	2.1	2.4
10A	June 1	152	1.0	2.5	58	3.6	4.2
10B	" 11	286	1.1	4.0	140	7.8	8.7
11	" 1	100	1.0	2.0	61	3.4	4.0
12	" 3	127	0.5	1.8	67	2.5	3.3
13	" 3	81	0.0	0.8	65	3.4	3.8
14	" 3	111	0.2	1.3	69	3.0	3.7
15	" 6	91	0.0	0.9	59	1.9	2.5
16	" 6	109	0.6	1.7	75	2.9	3.6
17	" 8	145	0.0	1.3	66	2.6	3.0
18	" 11	78	0.4	1.2	59	2.7	3.3
19	" 11	83	0.2	1.0	60	1.9	2.5
20	" 15	164	1.9	3.5	70	3.8	4.6
21	" 23	100	0.5	1.5	73	3.9	4.6
22	" 25	90	0.6	1.5	70	2.8	3.4
23	" 25	89	0.6	1.5	67	2.7	3.3
24	" 26	93	0.3	1.2	69	2.9	3.6
25	" 27	104	0.2	1.2	74	3.0	3.7
26	July 6	113	0.2	1.3	23	0.7	1.0
27A	" 6	158	0.0	1.5	81	3.8	4.6
27B	" 7	194	0.6	2.5	82	3.0	3.8
28	" 17	110	0.9	2.0	3	0.3	0.4
29	" 17	81	0.0	0.8	13	0.4	0.5
30	" 22	154	1.0	2.5	17	0.2	0.4
Mean.....		115	0.5	1.6	64	2.9	3.4
Median.....		102	0.5	1.5	68	2.9	3.6

The "uncorrected" values of "fructose" have not been corrected for the resorcinol reaction given by the glucose present.

tainty. Most of the results upon spinal fluid, however, were significant. There was a much greater concentration of fructose in spinal fluid than in blood in all the studies except two (Series 28 and 30) upon patients with meningitis. The glucose in the spinal fluid was low in the specimens in both these instances.

Since the analyses reported here were carried out exactly as were those upon the two cases of epidemic meningitis previously described (6), the two series may profitably be compared. When the fructose values are so compared, it is evident that most of the results in the present series, which contained many specimens approximately normal in composition, were much higher than were those found in the study of meningitis, in which the average of all the analyses, including those made upon specimens drawn after recovery and during remissions, was only 0.9 mg. per 100 cc. The difference between the two sets of figures might either be due to a difference between the clinical conditions of the patients or to some other factor. Because the glucose content of the specimens from the patients with meningitis was low (the average content was 23.9 mg. per 100 cc.), and because some parallelism was noted between glucose and fructose both in the meningitis patients and in the specimens reported in the preliminary note, the authors were inclined to feel that the variations in total sugar, which is largely glucose, might be directly related to these differences in fructose.

It seemed worth while to investigate the results obtained in the present study to determine to what extent the presence of such a relationship could be demonstrated in them. Statistical methods were used for this purpose. The correlation coefficient between the total sugar and the corrected fructose was calculated and found to be $+0.85 \pm 0.03$.

It seemed necessary to carry out further calculations to prove that this marked apparent correlation was significant. Since glucose itself gives a reaction with the resorcinol reagent, and since the determination of the glucose correction cannot be considered as wholly satisfactory, it was thought possible that the correlation might have been markedly influenced by measurements of the color from this source. However, the correlation coefficient between total glucose and uncorrected fructose values was $+0.91 \pm 0.02$, only slightly higher than that between glucose and fruc-

tose, and the correlation coefficient between total glucose and fructose from which twice the glucose correction was subtracted was also very high, $+0.78 \pm 0.05$. It seems safe to conclude, therefore, that the reaction of glucose did not greatly influence the value of the coefficient. It seemed desirable also to determine the degree of correlation between what may be called "true glucose" and fructose. This "true glucose" was calculated by subtracting from the total sugar the glucose equivalent of the fructose present. The correlation coefficient between these values and the fructose concentrations was $+0.80 \pm 0.04$, a value which did not differ significantly from that between "total glucose" and fructose. Although the parallelism between the two sugars is not exact, it is evident from these calculations that there is a definite, marked correlation between them.

Correlation coefficients between all of the other factors given in Table II were also calculated. The only ones which showed statistical significance were those which expressed the relationship between glucose and fructose in blood. The authors believe that this relationship was apparent rather than real. The coefficient between glucose and uncorrected fructose was $+0.89 \pm 0.025$, and that between glucose and corrected fructose $+0.50 \pm 0.09$. It seems probable that most, if not all, of this apparent correlation arose from the facts that the color measured in the filtrate was largely due to glucose, and that the measurements of both the fructose and the glucose correction were not very satisfactory.

The presence of fructose in spinal fluid in greater concentration than in the blood and the existence of a parallelism between the glucose and fructose concentrations in the spinal fluid can be explained in various ways. It seems improbable that the source of the fructose in spinal fluid was the preformed sugar in the blood, for in that case it would be almost necessary to assume that the sugar could enter but could not leave the spinal canal. If part of the glucose in spinal fluid were in some form which gives an intense reaction with the resorcinol reagent more readily than do solutions of crystalline glucose, the findings would be accounted for. This also seems improbable, because when normal spinal fluid was incubated with the cellular elements from meningitic fluid (6), the glucose, measured by the total reducing power, was almost wholly destroyed and the fructose but little affected. The "glucose" and

"fructose" in spinal fluid seem, therefore, to be separate compounds.

Since these two sugars are present in spinal fluid in concentrations which parallel each other quite closely, and since only one of them was present in blood in significant amounts, it seems probable that the fructose found in the fluid was formed from glucose. Such a change might be due to the formation of a hexosephosphoric acid ester; a synthesis of this type would resemble biological reactions known to take place (16). Great emphasis cannot be placed upon this explanation because the "fructose" seems to exist as the free sugar, and, therefore, it appears to be necessary to postulate a decomposition as well as a formation of the ester. While the authors feel that such an explanation is by no means inconceivable, they have not been able to obtain direct evidence in its favor.

Another mechanism by which the formation of fructose from glucose might be explained is through a rearrangement of the glucose molecule similar to that first described by Lobry de Bruyn and Van Ekenstein (17). While it can be shown that such a rearrangement takes place when glucose is allowed to stand with sodium hydroxide at room temperature, is incubated with sodium carbonate at 38°, or is boiled in neutral solution (18), the transformation could not be demonstrated at 38° at reactions such as occur in the spinal canal. Sterile glucose dissolved in $m/15$ NaHCO_3 and $m/15$ Na_2HPO_4 to give concentrations of 10 to 100 mg. per 100 cc. was repeatedly incubated 24 hours at 38°. No measurable formation of fructose occurred. These experiments show that the conversion of one sugar into the other through such a molecular change cannot be definitely assumed as an explanation of the presence of fructose in the spinal fluid. They do not, however, prove that the sugar is not formed in some such way.

A change of glucose into fructose through some reaction resembling the Lobry de Bruyn conversion, through an intermediate formation of a hexosephosphoric acid ester, or in some other way might take place within the free spinal fluid. From the negative results obtained in incubating glucose in weakly alkaline carbonate and phosphate solutions, it seems necessary to assume either that spinal fluid contains an enzyme which promotes the change of one sugar into the other, or that the glucose in spinal fluid is in some form which is more readily converted into fructose than

is ordinary glucose. A number of specimens of spinal fluid and of spinal fluid enriched with glucose have been incubated *in vitro* to test the truth of either of these hypotheses. A formation of fructose was sometimes noted in both types of experiments, but negative findings were as often encountered. The authors believe that the positive findings were as easily explained on the basis of an increased alkalinity which developed in the incubated specimens as by the presence of an enzyme in the spinal fluid or by the existence of some "active" form of glucose in the material. Neither hypothesis was entirely contradicted by the results, but certainly neither was definitely established by them.

It seems possible that formation of fructose from glucose, with or without the formation of an ester, might take place during the passage of the sugar through the membranes between the blood and spinal fluid. This seems to be an especially plausible hypothesis, since during the passage the sugar is presumably exposed to the action of cellular enzymes. Two observations seem to make it difficult to accept such an explanation. One is the fact that there was no significant correlation between the blood sugar and the spinal fluid fructose. The other is that low glucose and low fructose values occurred simultaneously in spinal fluid from meningitic patients. It is quite generally agreed (19) that the low glucose values found in such conditions arise at least in part from the action of enzymes in the leucocytes in spinal fluid, and it has been shown in a previous publication (6) that incubation of certain spinal fluid specimens rich in leucocytes may destroy much of the glucose without significantly affecting the fructose.

It is possible that a formation of fructose from glucose, either through one of the two chemical reactions suggested above or in some other way, might be brought about by the cells lining the spinal canal. It is difficult to establish definite proof in favor of such a hypothesis, and it seems less probable than does a change during the passage of sugar from the blood into the spinal fluid, but it seems to the authors to explain the presence of fructose in spinal fluid in greater concentration than in blood, the approximate, but not exact parallelism between glucose and fructose in the spinal fluid, the absence of correlation between blood glucose and spinal fluid fructose, and the frequent absence of significant increases in the fructose content when spinal fluid is incubated, at least as well as does any other.

Spinal fluid contains a substance having the properties of fructose. The substance was present in spinal fluid in higher concentration than in samples of blood taken at the same time. The concentration of the fructose paralleled the concentration of the total sugar in the spinal fluid. It seems probable that the fructose was formed from glucose, but, although various experiments were carried out to study the problem, the chemical nature of the change and the mechanism by which it might have been brought about were not determined.

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A SIMPLE METHOD FOR THE DETERMINATION OF ACETONE IN BLOOD AND URINE

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With the increasing use of volatile solvents in industry, simple and rapid methods for their detection in body fluids are becoming more important both from a medicolegal and a clinical point of view. Recently we described such a technique for the determination of ethyl alcohol in blood (1) and saliva (2), and the present paper is an extension of the same method for estimating acetone and other volatile ketones.

The reaction of acetone with Nessler's solution to form a cream-white precipitate is a familiar observation, and we have made use of this reaction for the quantitative detection of as little as 0.002 mg. of acetone in a 0.5 ml. sample of blood or urine. The acetone is first absorbed into a solution of sodium bisulfite which is then treated with the Nessler's solution; precipitates form, varying in intensity from a faint haze to definite turbidities, depending upon the amount of acetone present. These turbidities are finally compared against a set of standards made from known amounts of acetone. The ketone can be determined in the blood or urine without any preliminary protein precipitation, dilution, or distillation of acetone. The whole determination takes 20 minutes, and the actual manipulation less than 5 minutes.

Method

Reagents—

1. Sodium bisulfite, 5 per cent solution in water.
2. Nessler's solution (Koch and McMeekin's reagent (3)).
3. Sulfuric acid, 1:1 dilution by volume.
4. Standard acetone solution. A stock solution containing 0.1

mg. per ml. of acetone is prepared and checked gravimetrically by the method of Van Slyke (4). This solution retains its titer for about 2 weeks. The standard solutions used are made by a 1:10 dilution of this, so that 1 ml. contains 0.01 mg. This should be prepared daily.

Technique—The apparatus consists of a 50 ml. Erlenmeyer flask and a tightly fitting cork. Suspended from the under surface of the cork by means of a pin is a cm. length of cotton pencil. This pencil is a compressed roll of absorbent cotton, and is commonly supplied by many firms (Johnson and Johnson) to dentists under the name of dental rolls. It is best to use a pin about 6 cm. long, and with a large, beaded head. The pin is stuck first through the cotton plug and then into the cork.

The urine is made acid to Congo red with 1:1 sulfuric acid, and 0.5 ml. is pipetted onto the cotton roll. In the case of the blood, no previous acidification is necessary, and the 0.5 ml. sample may be used directly. 0.5 ml. of the 5 per cent sodium bisulfite is spread over the bottom of the flask, and, the cork is carefully and firmly inserted so as to allow the blood or urine sample to suspend about 1 cm. over the level of the bisulfite. The flask is heated in a boiling water bath for 15 minutes, and when cool, the cork and cotton roll are removed. 1 ml. of water is added, then 1 ml. of Nessler's solution, bringing the total volume to 2.5 ml. The solution is poured into a test-tube (6 inches \times 0.5 inch) and the amount of turbidity compared with the standard set.

The standards are prepared at the same time in similar test-tubes from 0, 0.002, 0.004 . . . 0.010 mg. of acetone, water to 1 ml., 0.5 ml. of 5 per cent sodium bisulfite, and 1 ml. of Nessler's solution. The full development of the turbidity takes 15 minutes, during which time the tubes should be shaken occasionally. If exposed as little as possible to the light, they keep for about 24 hours. When abnormal amounts of acetone are suspected in the blood or urine, a stronger set of standards containing 0, 0.005, 0.010 . . . 0.050 mg. of acetone should be prepared.

Results

Ten blank determinations with water alone on the cotton roll failed to reveal any acetone value. We then applied the method

to the estimation of acetone and acetoacetic acid in the blood and urine of normal individuals, and obtained the following values.¹

	No. of observations	Acetone mg. per cent
Blood.....	30	0.3-2.0
Urine.....	50	0.2-2.5

Recovery experiments were also carried out on samples of human blood and urine with the results shown in Table I.

TABLE I
Recovery of Acetone from Blood and Urine

Ten experiments were performed in each case.

	Acetone added to 100 ml.	Acetone found in 100 ml. (blank corrected)	Recovery
	mg.	mg.*	per cent
Blood	0.50	0.45 \pm 0.04	90
	1.00	1.10 \pm 0.05	110
	2.00	2.20 \pm 0.10	110
	4.00	3.80 \pm 0.20	95
	8.00	8.50 \pm 0.50	106
Urine	0.50	0.46 \pm 0.04	94
	1.00	0.95 \pm 0.05	95
	2.00	2.20 \pm 0.08	110
	4.00	4.20 \pm 0.25	105
	8.00	7.60 \pm 0.45	95

* \pm average deviation.

Table II gives the values obtained by this method as compared with those obtained by that of Van Slyke (4) and of Ravin's modification of the Behre and Benedict technique (5).

DISCUSSION

It is to be noted that this method is by no means specific for acetone, for other ketones volatile in sufficient quantity under the

¹ These, and many of the following experiments, were carried out by using filter paper rolls instead of the cotton plugs. The preparation of these filter papers is described in a previous paper (1); twenty-five blank determinations with these papers similarly failed to show any acetone values, so the papers are quite safe to use, although less convenient.

conditions of the experiment are absorbed by the bisulfite and form similar precipitates with the Nessler's solution. We have found this to be so with butanone, and this may be quantitatively recovered from blood or urine in amounts as little as 0.002 mg. from 0.5 ml. samples.

There are, however, interfering substances which must be considered, principally ammonia and aldehydes. Only in the case of the urine is the former important, and unless the specimen is sufficiently acidified, the volatile ammonia dissolves in the bisulfite solution, and a heavy orange precipitate results upon the addition of the Nessler's solution. Fabre (6) and Supniewski (7) have found acetaldehyde to be the chief aldehyde of the blood,

TABLE II
Determination of Acetone and Acetoacetic Acid

Urine No	This method	Van Slyke	Behre-Benedict
	mg per cent	mg per cent	mg. per cen ^o
1	1 60	1 45	
2	2 00	1 80	
3	1 80	1 70	
4	1 00		1 20
5	0 80		0 90
6	1 50		1 60
7	1 10	1 20	1 05
8	5 90	6 05	6 00
9	5 40	5 30	5 25
10	5 00	5 15	5 10

and to occur in normal concentrations of from 0.02 to 0.06 mg. per cent. Formaldehyde may also exist in larger amounts in the urine after certain medication (urotropine). Ordinarily the aldehydes are differentiated by this reaction from the ketones by the character of the precipitate formed: the ketones cause cream-white turbidities, while equal amounts of aldehyde form dark, metallic gray precipitates, so that when they occur in the urine or blood in excess their presence is readily manifested. However, in their normal concentrations, in the presence of a large excess of ketone, they must figure in the error of the determination, and may thus occasionally form as much as 15 per cent of the "found ketone" before lending a grayish color to the precipitate. This last is

obtained when the acetone is at the lower limit and the acetaldehyde at the upper limit of their normal ranges; usually the aldehyde will form only about 5 per cent of the estimated ketone.

Methyl and ethyl alcohols, and ethyl and butyl acetates, do not react with the Nessler's solution except in relatively large concentrations (about 1 per cent).

For the complete absorption of the ketones we have found it necessary to use a large excess of the bisulfite reagent. The dissociation constant of the acetone-bisulfite complex is relatively large, and it is only by means of the excess that an adequate absorption is obtained (8).

In these determinations we have found it necessary to use fresh samples of blood and urine, for as much as 20 per cent of the acetone is destroyed within 1 day. This is probably in a large measure due to bacterial decomposition, for when toluene is used to preserve the specimen, only about 5 to 10 per cent of the ketone is found to have disappeared. This, again, may be due to the acetone passing into solution in the toluene in which it is quite soluble (the partition coefficient of acetone between water and toluene is 2:1). Thus it is desirable to avoid using an excess of toluene as a preservative.

I wish to express my gratitude to Dr. I. Greenwald for his many helpful suggestions and very kind interest in this problem.

SUMMARY

A rapid and simplified method for the determination of ketones in blood and urine is presented.

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CYSTINURIA*

V. THE METABOLISM OF CASEIN AND LACTALBUMIN

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In previous investigations (1, 2) when cystine, cysteine, and methionine were fed to cystinuric patients, it was observed that cysteine and methionine yielded considerable amounts of extra cystine in the urine, in contrast to cystine, which was oxidized to inorganic sulfate. These findings were recently confirmed by Lewis, Brown, and White (3).

It was concluded from these investigations that the cystine of the urine in cystinuria is derived in part or in whole from that portion of the protein sulfur which is present in the protein molecule in the form of methionine. It was therefore of interest to investigate the fate of protein sulfur in the cystinuric organism by superimposing upon the basal diet several proteins containing methionine and cystine in various proportions. Casein and lactalbumin were chosen for these experiments. The former contains considerable amounts of methionine but little cystine, while the sulfur in the latter protein is distributed about equally between methionine and cystine. In order to administer about equal amounts of sulfur, 200 gm. of casein and 100 gm. of lactalbumin were fed.

The experiments reported below indicate that cystine and methionine, fed as casein and lactalbumin, were catabolized in the same way, both qualitatively and quantitatively, as when administered in the form of the free amino acids.

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EXPERIMENTAL

Case History and Diet—The cystinuric patient was the one on whom the previous studies were carried out (1, 4); the experimental periods on this patient are from now on designated by the letter A. He has been maintained at home on essentially the same weighed diet to which he rigidly adheres, as can be seen from the nitrogen and sulfur figures of the control periods (Table II, Periods A23, A25, A27.) Up to the present (December, 1936),

TABLE I
Analysis of Casein and Lactalbumin

	Casein*	Lactalbumin†
	<i>per cent</i>	<i>per cent</i>
Nitrogen .	14.8	14.8
Sulfur	0.78	1.42
Methionine S	0.68	0.59
Cystine S	0.10	0.83
Methionine	3.2	2.8
Cystine	0.37	3.1
	<i>ratio</i>	<i>ratio</i>
N:S	19	10
N to methionine S	22	25
" " cystine S	150	18
Methionine S to cystine S	7	0.7
" " to cystine	9	1
	<i>per cent of total S</i>	<i>per cent of total S</i>
Methionine S	87	42
Cystine S.	13	58

* Moisture, 5 per cent.

† Moisture, 3 per cent.

there has been no evidence of new stone formation, as determined by frequent x-ray observations.

Analysis of Casein and Lactalbumin—The casein and lactalbumin were Labco products obtained through the courtesy of Dr. G. C. Supplee. The proteins were analyzed for the various constituents summarized in Table I. A number of methods were used in the analysis for sulfur, cystine, and methionine; some of the details will be reported in a separate communication (5). The figure

reported in Table I for casein sulfur is the average of several determinations by the Parr bomb, while that for lactalbumin sulfur is the average of several estimations by the Pregl method. The determination of cystine with the Sullivan method and with the Folin photometric procedure¹ (6) yielded identical results (for details *cf.* (5)). Methionine was determined according to the volatile iodide method of Baernstein (7), except for some minor modifications (5). The methionine values reported in Table I are corrected for methyl mercaptan formation (5). Although the total sulfur in these proteins has apparently been accounted for by cystine and methionine, it is possible, according to Blumenthal and Clarke (8), that there are present in casein and lactalbumin very small quantities of unknown sulfur compounds, in amounts which we believe are metabolically insignificant. It should be noted that the N:S ratio of casein is 19 and that of lactalbumin 10, while the methionine to cystine ratios are 9 and 1 respectively. A still greater difference is shown by the ratios of N to methionine S and N to cystine S.

Methods—The values recorded in Table II were determined by the methods indicated and discussed in previous publications (1, 4).

Metabolic Observations—The excretion of the various urinary constituents (see Table II) was constant during the control periods. Throughout the experiment there was little change in the volume, pH, and specific gravity of the urine and in the excretion of undetermined nitrogen. During the experimental periods there was a slight rise in the excretion of ammonia and of amino acid nitrogen. These values were omitted from Table II. Likewise omitted were the figures for cystine obtained by the Folin-Marenzi and Sullivan methods, having no special significance in this experiment (for discussion of the results yielded by the various cystine methods *cf.* (1, 4)). Determinations for homocystine, carried out by the modification of the photometric procedure mentioned previously (*cf.* (4, 6)), were negative throughout

¹ For these determinations lactalbumin was hydrolyzed with HCl and also with H₂SO₄. Cysteine was absent in the H₂SO₄ hydrolysates, while the method indicated 0.3 per cent cysteine in the HCl hydrolysates. Even if this quantity of cysteine were present in lactalbumin, it would be too small to be of significance, metabolically and with respect to the calculations presented in Table VII.

TABLE II
Metabolic Observations

Period No.	Date	Substance fed	Urine											
			Nitrogen		Creatinine		Sulfur					Cystine		
			Total	Urea	Preformed	Total	Total	Inorganic SO ₄	Etihæral SO ₄	Total	Cystine	Undetermined	Polin photo-metric	Lugg-Sullivan
		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
A23*	1934 July 22-24	95	5.8	4.3	1.20	1.33	0.54	0.25	0.02	0.27	0.20	0.07	0.75	0.76
A24	" 25	95	12.2	10.1	1.18	1.28	0.70	0.33	0.02	0.35	0.27	0.08	1.01	0.94
A24a	" 26		14.9	12.9	1.24	1.38	0.81	0.37	0.01	0.43	0.33	0.10	1.23	1.26
	" 27		8.2	5.8	1.16	1.36	0.65	0.29	0.02	0.34	0.26	0.08	0.98	1.03
	" 28		6.4	4.6	1.24	1.28	0.57	0.29	0.02	0.26	0.21	0.05	0.79	0.73
	" 29		7.5	5.5	1.25	1.34	0.62	0.30	0.02	0.30	0.24	0.06	0.87	0.78
A25*	" 30-31		6.5	4.7	1.16	1.28	0.57	0.29	0.02	0.26	0.20	0.06	0.75	0.68
A26	Aug. 1	Lactalbumin	8.1	6.0	1.19	1.38	0.81	0.48	0.03	0.30	0.22	0.08	0.81	0.80
A26a	" 2		8.2	6.5	1.13	1.32	0.84	0.48	0.04	0.32	0.25	0.07	0.93	0.88
	" 3		7.1	5.2	1.13	1.26	0.60	0.29	0.02	0.29	0.23	0.06	0.85	0.82
	" 4		6.1	4.5	1.23	1.35	0.55	0.26	0.02	0.27	0.21	0.06	0.80	0.85
	" 5		6.1	4.5	1.24	1.42	0.56	0.26	0.02	0.28	0.22	0.06	0.83	0.81
A27*	" 6-7		5.6	3.7	1.09	1.24	0.51	0.24	0.02	0.26	0.20	0.06	0.75	0.75

the experiment. Tests for sulfhydryl compounds in the urine were always negative.

Creatinine Excretion—Both during the control and experimental periods there were only minor variations in the excretion of pre-formed creatinine, indicating conditions satisfactory for metabolic experimentation. It is of interest to note that ingestion of 200 gm. of casein did not result in a striking rise of the creatinine output, in agreement with observations of many investigators, but contrary to a report by Beard and Barnes (9). Furthermore, we have fed large amounts of casein to two normal human subjects without observing any major rise in the creatinine excretion.

Casein (Periods A24 and A24a)—It can be seen from Table II that the ingestion of 190 gm. of casein resulted in a considerable increase in the excretion of cystine and in a small rise in that of inorganic sulfates. The excretion of the extra sulfur continued for several days in the adjustment period. The excretion of extra nitrogen in the feeding and adjustment periods amounted to about 19 gm. Smaller amounts of extra nitrogen and of extra inorganic sulfate continued to appear in the after period (No. A25), although the cystine excretion had already returned to the basal level.

Lactalbumin (Periods A26 and A26a)—It can be seen from Table II that the ingestion of 97 gm. of lactalbumin resulted in a considerable increase in the excretion of inorganic sulfates and in a small rise in that of cystine. The excretion of the extra sulfur continued in the adjustment period, the greater part appearing on the 1st day (Period A26a). The excretion of extra nitrogen in the feeding and adjustment periods amounted to about 5 gm. In contrast to the casein experiment no extra sulfur and nitrogen appeared in the after period (A27).

Results

A summary and an analysis of the data are given in Tables III to VII. The method of calculation of the extra nitrogen, extra sulfur, and of the partition of the extra sulfur has been discussed in Paper II of this series (*cf.* (1) p. 77). The values for extra N, extra total S, extra cystine, etc., were obtained as described previously and as illustrated by the following example

Cystine, Periods A24 to A24a (5 days)	gm. = 4.88 minus
“ daily average, Periods A23 and A25, $\times 5$	= 3.75
Extra cystine, Periods A24 to A24a	= 1.13

It can be seen from Table III that 18.1 gm. of extra N, or 67 per cent of the amount fed, appeared in the urine in the casein experiment, and 5.1 gm. of extra N, or only 35 per cent, were excreted in the lactalbumin period. Although twice as much casein was fed as lactalbumin, the extra total S excreted was

TABLE III

Extra Nitrogen, Total Extra Sulfur, and Extra Cystine in Experimental Periods

Period No.	Substance fed		Amount of extra N				Amount of extra S			Extra cystine excreted
			Fed		Excreted		Fed		Excreted	
		gm.	gm.	gm.	per cent	gm.	gm.	per cent	gm.	
A24-A24a	Casein	190	28 1	18 7	67	1 48	0 60	41	1 13	
A26-A26a	Lactalbumin	97	14 4	5 1	35	1 38	0 66	48	0 47	

TABLE IV

Nitrogen, Sulfur, Methionine, and Cystine Ratios in Experimental Periods

	Casein, Periods A24-A24a						Lactalbumin, Periods A26 A26a					
	N	S	N S	N to metho- nine S	N to cystine S	Methionine to cystine	N	S	N S	N to metho- nine S	N to cystine S	Methionine to cystine
	gm.	gm.					gm.	gm.				
Fed	28 1	1 48	19	22	150	9	14 4	1 38	10	25	18	1
Catabolized	18 7	0 60	30	36*	230*	9*	5 1	0 66	8	18*	13*	1*
"Stored"	9 4	0 88	11	?	?	?	9 3	0 72	13	?	?	?
				(12)†	(85)†	(9)†				(31)†	(22)†	(1)†

* Cf. "Results."

† Calculated for the non-catabolized residues of casein and lactalbumin, the actual amino acid composition of the "stored" material remaining unknown.

approximately the same; i.e., 0.60 and 0.66 gm. respectively. The extra total S accounted for 41 and 48 per cent respectively of the sulfur ingested as casein and lactalbumin.

In Table IV the figures for nitrogen and sulfur, fed, catabolized, and "stored" in the experimental periods, are tabulated and the corresponding ratios calculated. The values given for N and S

"stored" were obtained by subtracting the amounts of N and S catabolized (*i.e.* excreted in the urine as extra N and S) from those administered. It should be noticed that the N:S ratio of the casein "stored" is much lower and the N:S of the casein "catabolized" much higher than the N:S ratio of the original protein, these ratios being 10, 30, and 19 respectively. The N:S ratios of lactalbumin fed, catabolized, and "stored" are, on the other hand, much more constant, being 10, 8, and 13 respectively. The significance of the relatively close agreement of the N:S ratios of the material "stored" after the feeding of casein and of lactalbumin is difficult to evaluate, since the actual amino acid composition of the "stored" material remains unknown.

TABLE V

Partition of Extra Sulfur in Experimental Periods and after l-Cystine and dl-Methionine

Period No.	Substance	Extra S excreted as					
		Total S	Inorganic SO ₄ -S		Cystine S		Undetermined neutral S
		gm.	gm.	per cent	gm.	per cent	gm. per cent
A24-A24a	Casein	0 60	0 25	42	0 30	50	0 04 7
A26-A26a	Lactalbumin	0 66	0 44	67	0 13	20	0 06 9
A10-A10a	l-Cystine*			94		0	7
A14-A14a	dl-Methionine*			33		51	15

*Cf. (1) Fig. 1.

In the casein experiment 1.13 gm. of extra cystine were excreted as compared to only 0.47 gm. in the lactalbumin periods (Table III). The extra cystine accounted for 50 per cent of the extra sulfur excreted after the feeding of casein, but for only 20 per cent after lactalbumin (Table V). Correspondingly, in the casein period smaller amounts of extra inorganic sulfate (0.25 gm.) were found than in the lactalbumin period (0.44 gm.), accounting for 42 per cent of the extra sulfur in the case of the former and for 67 per cent in the latter (Table V).

Although the amount of sulfur fed and catabolized (*i.e.* extra S excreted) was quite similar in both the casein and lactalbumin experiments, the differences in the partition of the extra sulfur

are striking. The protein (casein), which contains a large amount of methionine, but little cystine, yielded the larger amounts of extra cystine and smaller quantities of extra inorganic sulfate, while the protein (lactalbumin), in which the sulfur is about equally distributed between methionine and cystine, gave large amounts of extra inorganic sulfate and smaller quantities of extra cystine (see Tables III and V). These findings are, in a general way, in agreement with our previous experiments (1) on the feeding of cystine and methionine² to this cystinuric patient, which experiments showed that cystine was almost completely oxidized to inorganic sulfate, while methionine was largely excreted as extra cystine, only a small part being oxidized (see Table V).

It, therefore, appears that cystine and methionine, fed as casein and lactalbumin, were handled in approximately the same way

TABLE VI
Total S, Cystine S, and Methionine S, Fed and Catabolized

Period No.	Substance	S fed			S catabolized		
		Total	Cystine	Methionine	Total	Cystine*	Methionine*
		gm.	gm.	gm.	gm.	gm.	gm.
A24-A24a	Casein	1.48	0.19	1.29	0.60	0.08	0.52
A26-A26a	Lactalbumin	1.38	0.80	0.58	0.66	0.38	0.28

* Calculated, cf. "Results."

as when administered in the form of the free amino acids. This point can be more conclusively demonstrated by a detailed evaluation of the data, based upon the assumption that the total sulfur catabolized was derived from methionine and cystine in the same ratios as they are present in the two proteins. The calculations necessary for this evaluation are given in Tables VI and VII. Table VI, under the heading sulfur fed, shows the amount of S in the two proteins as total S and in terms of cystine and methionine S, based on the analytical figures given in Table I. The value for total S catabolized was taken from Table III. The total

² In these experiments *dl*-methionine was used. The results of the protein feeding can be compared with the previous experiments because it is known that *d*- and *l*-methionine are utilized equally well for purposes of growth.

TABLE VII
Partition of Extra Sulfur Calculated and Found

Period No.	Partition of extra S calculated from	Inorganic SO ₄ -S gm.	Undeter- mined neutral S gm.	Cystine S gm.	Cystine gm.
Casein, A24-A24a	0.08 gm. cystine S catabolized to	0.08	0.0	0.0	0.0
	+0.52 gm. methionine S catabolized to	0.17	0.08	0.27	1.01
	Partition of extra S calculated	0.25	0.08	0.27	1.01
Lactalbumin, A26-A26a	" " " found (cf. Table V)	0.25	0.04	0.30	1.13
	0.38 gm. cystine S catabolized to	0.36	0.02	0.0	0.0
	+0.28 gm. methionine S catabolized to	0.09	0.04	0.14	0.52
	Partition of extra S calculated	0.45	0.06	0.14	0.52
	" " " found (cf. Table V)	0.44	0.06	0.13	0.47

S catabolized amounted to 41 and to 48 per cent respectively of the total S fed. It was, therefore, calculated that 41 per cent of the cystine and methionine fed as casein and 48 per cent of the sulfur amino acids fed as lactalbumin were catabolized. These values are given in Table VI under cystine S and methionine S catabolized.

In Table VII the partition of extra sulfur was calculated from the data in Tables V and VI, as illustrated by the following example. In Table VI it was shown that 0.28 gm. of methionine S was catabolized in the lactalbumin experiment; according to Table V 0.28 gm. of methionine S should yield 0.09 gm. of inorganic sulfate S (33 per cent), 0.04 gm. of undetermined neutral S (15 per cent), and 0.14 gm. of cystine S (51 per cent). The other data given in Table VII were calculated in the same way. It will be seen in Table VII that the partition of the extra S calculated as described above is in remarkable agreement with that actually found. This indicates the validity of the assumption made above; *i.e.*, the ratio of catabolized methionine to catabolized cystine is identical with the methionine to cystine ratio of the original protein (*cf.* Table IV).

It was possible, therefore, to calculate an N to methionine S and an N to cystine S ratio for the catabolized portion of the two proteins as given in Table IV. In Table IV, under the heading protein "Stored," the N:S ratio alone is that of the storage protein; the ratios N to methionine S, N to cystine S, and methionine to cystine (given in parentheses) are calculated for the non-catabolized residues of casein and lactalbumin, the actual amino acid composition of the "stored" material remaining unknown.

DISCUSSION

The results of the experiments with casein and lactalbumin reported in this paper are in agreement with our previous suggestion that one of the pathways of methionine catabolism³ is its

³ The possibility of a second metabolically important pathway of methionine catabolism is shown, we believe, by the recent experiments of Rose and his coworkers (10). They have found that some methionine is necessary for growth, although a considerable portion of the organism's need for sulfur can be supplied by cystine. Cystine, on the other hand, is not required for growth when ample amounts of methionine are given. Since

conversion into cysteine (for a possible mechanism *cf.* (11)) and that the cystine excreted in cystinuria is derived mainly from dietary methionine. It has been claimed on the basis of feeding experiments with rabbits (12) and as a result of *in vitro* experiments (13) that the first step in the catabolism of cystine is its reduction to cysteine, while our experiments in cystinuria (1) showed that cystine is apparently catabolized by the cystinuric without such a reduction. It is therefore, interesting to note that during the catabolism of both casein and lactalbumin no appreciable reduction of cystine to cysteine seemed to have occurred, since all of the extra cystine excreted was apparently derived from the catabolism of methionine.⁴ The data also indicate that there are no metabolically significant amounts of sulfur present in casein or in lactalbumin which cannot be accounted for by either methionine⁵ or cystine. It thus appears that, under the conditions of these experiments, cystine and methionine, fed as constituents of casein and of lactalbumin, were catabolized both qualitatively and quantitatively in the same way as when they were administered in the form of the free amino acids.

Further implications of these data and their bearing on certain phases of intermediary protein metabolism will be discussed at a later date.

We are indebted to the patient, Mr. L. P., for his cooperation which made these studies possible.

SUMMARY

1. The distribution of the sulfur was determined in a preparation of casein and in one of lactalbumin. The methionine to cystine ratio of the casein was 9 and that of the lactalbumin 1.

we have recently shown (1) that cystine and cysteine are not entirely interchangeable, it will be necessary to demonstrate the failure of cysteine and glutathione to support growth in the absence of methionine in order to establish definitely this second pathway of methionine metabolism.

⁴ Cysteine yields extra cystine in the urine (1). If an appreciable amount of cystine had been reduced during the catabolism, the values found for the partition of the extra sulfur would not check with the figures calculated in Table VII.

⁵ The results, therefore, support the validity of Baernstein's method for the determination of methionine.

2. The metabolism of casein and of lactalbumin was investigated in a case of cystinuria.

3. Under the conditions of the experiments, methionine and cystine fed as constituents of casein and lactalbumin were catabolized both qualitatively and quantitatively in the same way as when they were administered in the form of the free amino acids.

4. The total S catabolized was accounted for in terms of methionine and cystine. It was shown that methionine and cystine were catabolized in the same ratios in which they are present in casein and in lactalbumin.

5. The results are in agreement with previous suggestions that one of the pathways of methionine catabolism is its conversion into cysteine and that the cystine excreted in cystinuria is derived mainly from dietary methionine.

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CYSTINURIA*

VI. THE METABOLISM OF THE HYDROXY ANALOGUE OF METHIONINE (*dl*- α -HYDROXY- γ -METHIOBUTYRIC ACID)

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In previous experiments on cystinuric patients it was shown that methionine, cysteine, and homocysteine yield extra cystine in the urine (1-3). It was therefore of interest to investigate the fate, in the cystinuric organism, of other substances closely related to methionine. In the present communication we have tested the behavior of *dl*- α -hydroxy- γ -methiobutyric acid. It will be seen that this compound is only partially oxidized to inorganic sulfate but largely excreted as extra cystine and undetermined neutral sulfur.

EXPERIMENTAL

Case History, Diet, and Methods

The experiments were carried out on patient A (*cf.* (4)) under the same dietary conditions as were mentioned in Paper V (4). The methods used for the determination of the various urinary constituents were indicated in our previous publications ((1, 2); *cf.* (5)). The hydroxy analogue of methionine was prepared according to the method of Block and Jackson (6) as its zinc salt. The compound was free from N and melted at 250° (uncorrected) and gave the following analysis.

$C_{10}H_{18}O_6S_2Zn \cdot H_2O$	Calculated.	S 16.8, Zn 17.1
	Found.	" 16.9, " 16.9

* Aided in part by Grant 385 from the Committee on Scientific Research, American Medical Association.

The analysis for sulfur was satisfactory by the Parr bomb method; the Benedict-Denis method yielded low results (about 80 per cent).

The free hydroxy acid was not obtained in crystalline form but fed to the patient in aqueous solution, the greater part of the zinc having been removed by H_2S .

Metabolic Observations and Results

It can be seen that the excretion of the various urinary constituents reported in Table I was constant during the control periods.

TABLE I
Metabolic Observations

Period No	Date	α -Hydroxy γ -methiobutyric acid fed	Urine												
			Nitro- gen		Creatinine		Sulfur						Cystine		
			Total	Urea	Preformed	Total	Total	Inorganic SO ₄	Etheral SO ₄	Neutral			Photometric	Lugg-Sullivan	
										Total	Cystine	Undetermined			
	1935	gm.	gm	gm	gm	gm.	gm	gm	gm	gm	gm	gm	gm	gm	gm
A37*	July 29-30		7 4	5 6	1 31	1 43	0 69	0 32	0 04	0 33	0 22	0 11	0 83	0 82	
	" 31		2 0	7 1	5 0	1 29	1 44	0 77	0 33	0 03	0 41	0 27	0 14	1 00	1 06
A38	Aug 1		2 0	7 5	5 1	1 42	1 58	0 93	0 41	0 03	0 49	0 33	0 16	1 24	1 28
	" 2		4 0	7 2	5 0	1 37	1 49	1 13	0 45	0 03	0 65	0 35	0 30	1 32	1 40
A38a	" 3		6 8	4 8	1 33	1 48	0 81	0 34	0 02	0 45	0 30	0 15	1 11	1 25	
	" 4		7 3	5 4	1 35	1 45	0 72	0 30	0 02	0 40	0 26	0 14	0 96	1 05	
	" 5		7 3	5 3	1 34	1 47	0 75	0 32	0 04	0 39	0 25	0 14	0 93	0 95	
A39*	" 6-7		7 2	5 2	1 32	1 47	0 66	0 31	0 04	0 31	0 23	0 08	0 87	0 89	

* Average per day

Throughout the experimental period there was little change in the volume, pH, and specific gravity of the urine and in the excretion of ammonia and undetermined nitrogen. These values were omitted from Table I. Likewise omitted were the figures for cystine obtained by the Folin-Marenzi method, having no special significance in this experiment (for discussion of the results yielded by the various cystine methods cf. (1, 2)). Determinations for homo-

cystine carried out by a modification of the photometric procedure mentioned previously (2, 5) were negative throughout the experiment. Likewise negative were tests for sulfhydryl compounds in the urine.

During both the control and experimental periods there were only minor variations in the excretion of preformed creatinine, indicating conditions satisfactory for metabolic experimentation.

The results given in Table I show that the ingestion of 8 gm. of α -hydroxy- γ -methiobutyric acid resulted in only a small rise in the output of inorganic sulfate. The excretion of cystine was increased considerably, reaching almost double that of the control level. The increased excretion continued for several days in the adjustment period (No. A38a). The excretion of undetermined neutral sulfur was considerably increased.

TABLE II

Extra S, Extra Cystine, and Partition of Extra S in Experimental Periods after Feeding of dl- α -Hydroxy- γ -Methiobutyric Acid

Period No.	Amount of extra S			Extra cystine excreted	Extra S excreted as		
	Fed	Excreted			Inorganic SO ₄ -S	Cystine S	Undetermined neutral S
		gm.	gm.				
A38-A38a	1 71	1 06	62	1 46	0 26	0 31	0 46

A summary and an analysis of the data are given in Tables II and III. The method of calculation of the extra nitrogen, extra sulfur, and the partition of the extra sulfur has been discussed previously (1-4). It will be seen from Table II that 1.06 gm. of extra sulfur, i.e. 62 per cent of the amount fed, were excreted in the experimental period.¹ Extra cystine amounted to 1.5 gm. Table III shows that 25 per cent of the extra S is inorganic sulfate, while cystine S and undetermined neutral S account for 29 and 43 per cent respectively.¹

¹ It should be noted that the determination for total S in the urine was carried out by the Benedict method, which, as pointed out above, gives somewhat low results with hydroxymethiobutyric acid. The figures for total S and undetermined neutral S, given in Table II, are, therefore, somewhat low, if the greater part of the undetermined neutral S is accounted for by the excretion of the unchanged hydroxy acid.

It is interesting to note that the partition of the extra sulfur in this experiment differs somewhat from that observed after the feeding of *dl*-methionine (1) and of *dl*-homocysteine (2). It can be seen from Table III that in the *dl*- α -hydroxy- γ -methiobutyric acid experiment a greater portion of the extra sulfur was accounted for by undetermined neutral S than in the methionine and homocysteine experiments. This undetermined neutral S was *homocystine* S after the ingestion of *homocysteine* (2), and probably unchanged methionine³ in Periods A14 and A14a (methionine feeding). It is, therefore, not unreasonable to assume that most of the undetermined neutral S in Periods A38 and A38a was due to the excretion of the unchanged hydroxy compound. It is

TABLE III

Partition of Extra Sulfur after Feeding dl-Methionine, dl-Homocysteine,† and dl- α -Hydroxy- γ -Methiobutyric Acid*

Period No.	Substance fed	Inorganic sulfate S	Cystine S	Undetermined neutral S
		per cent	per cent	per cent
A14-A14a	<i>dl</i> -Methionine	33	51	15
A28-A28a	<i>dl</i> -Homocysteine	27	50	24
A38-A38a	<i>dl</i> - α -Hydroxy- γ -methiobutyric acid	25	29	43

* Cf. (1) Fig. 1.

† Cf. (2) Fig. 1.

intended to investigate this aspect by feeding experiments with the optical isomers of α -hydroxy- γ -methiobutyric acid.

Regarding the excretion of extra cystine following the administration of *dl*- α -hydroxy- γ -methiobutyric acid, we are inclined to believe that this finding indicates a conversion of the hydroxy analogue of methionine into cysteine.

DISCUSSION

It is known that certain of the essential amino acids can be replaced for purposes of growth by their corresponding hydroxy or

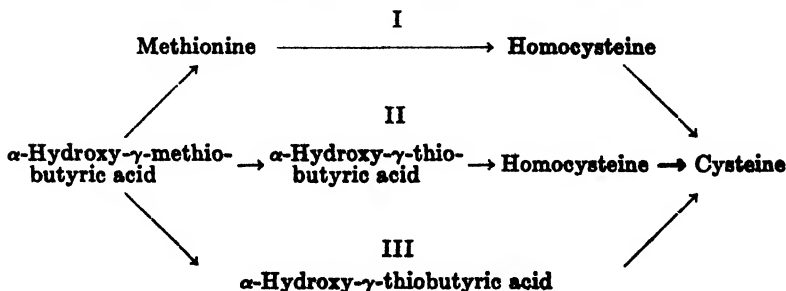
³ Andrews and Randall (7) reported the excretion of unchanged methionine by a cystinuric subject following the administration of *dl*-methionine (as determined by applying the Baernstein method to dried urine).

ketonic analogues. Thus *dl*-imidazolelactic acid is capable of replacing histidine (8) and *dl*-indolelactic acid (9, 10) and indolepyruvic acid (11) will substitute for tryptophane. On the other hand, cystine (12) and lysine (13) cannot be replaced by their hydroxy derivatives. It has been reported by Block and Jackson (6), in a preliminary note, that the zinc salt of *dl*- α -hydroxy- γ -methiobutyric acid is able to promote the growth of rats on a sulfur-deficient ration. Similar results were obtained in this laboratory (14). Akobe (15) recently prepared *d*- and *l*- α -hydroxy- γ -methiobutyric acid and claimed that both optical isomers have growth-promoting action with rats on the cystine- and methionine-poor diet recently devised by Kotake and his coworkers (16).

Such observations are frequently interpreted as indicating that the essential amino acid has been synthesized from its analogue during the course of metabolism. However, it is also possible that no actual synthesis occurred, but that the growth response is due to an intermediate compound common to the essential amino acid and its analogue. Growth experiments, without analysis of the whole carcass, do not permit us to decide between these two possibilities.³

In the light of our previous findings in cystinuria, the results reported in this paper and the growth-promoting action of α -hydroxy- γ -methiobutyric acid may indicate that methionine and its hydroxy analogue have a common intermediate, namely cysteine. The experiments do not permit us to differentiate between

Catabolism of α -Hydroxy- γ -Methiobutyric Acid



³ In this connection it is interesting to note that Conrad and Berg (17) have recently shown that *d*-histidine undergoes transformation into its natural *l* isomer in the growing rat.

the three possibilities with respect to the conversion of the hydroxy compound into cysteine, presented in the diagram.

In Reaction I methionine is synthesized, in Reaction II demethylation of the hydroxy compound is followed by synthesis of homocysteine, while in Reaction III it is assumed that the demethylated hydroxy derivative is converted into cysteine without synthesis of homocysteine. In regard to the possibility of Reactions II and III, it is of interest to note that when the zinc salt of α -hydroxy- γ -methiobutyric acid was shaken in the Warburg apparatus with kidney slices, a certain amount of demethylation took place, as indicated by a positive nitroprusside test. The reaction obtained was similar to that given by methionine treated under the same conditions (*cf.* (18)).

The experiments reported in this paper show that in the cystinuric a non-nitrogenous compound, α -hydroxy- γ -methiobutyric acid, can be catabolized to yield an amino acid which is, at least in part, wasted in the urine. These experiments are interpreted as furnishing additional evidence that one of the pathways of methionine catabolism may be its conversion into cysteine. The results are compatible with the working hypothesis recently suggested for this conversion (19).

SUMMARY

1. The metabolism of the hydroxy analogue of methionine, *dl*- α -hydroxy- γ -methiobutyric acid, was investigated in a cystinuric individual.

2. The compound is only partially oxidized to inorganic sulfate, but largely excreted as extra cystine and extra undetermined neutral sulfur. The compound supports growth of rats on a sulfur-deficient diet.

3. The results are in agreement with the hypothesis that one of the pathways of methionine catabolism is its conversion into cysteine and are compatible with the mechanism suggested for this conversion.

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CYSTINURIA*

VII. THE METABOLISM OF S-METHYLCYSTEINE, OF γ -THIOBUTYRIC ACID, AND OF γ,γ' -DITHIODIBUTYRIC ACID

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In continuation of our investigations on the metabolism of sulfur compounds in cystinuria, the fate of S-methylcysteine, of γ -thiobutyric acid, and of γ,γ' -dithiodibutyric acid was studied. The metabolism of the first substance was also investigated in a normal human being.

These compounds, although chemically related to cysteine and methionine, did not cause an increase in the excretion of cystine by the cystinuric. It is interesting to note that following the administration of γ -thiobutyric acid, no sulfhydryl groups could be detected in the urine. This thiol, like other —SH derivatives is apparently excreted by the cystinuric as an S—S compound.

EXPERIMENTAL

Case History, Diet, and Methods—The experiments were carried out on patient A (cf. (1)) under the same dietary conditions as mentioned in Paper V of this series. The methods used for the determination of the various urinary constituents were indicated in previous publications (1-4).

The normal control was a 26 year-old male (N), weighing 73 kilos. During the experimental periods he was maintained on a weighed, meat-free diet, of constant N and S content.

Preparation of Sulfur Compounds—S-Methylcysteine was prepared in a way similar to that described by Clarke and Inouye (5)

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for S-ethylcysteine. After removal of tin and of methyl alcohol, the compound was precipitated with copper carbonate and the copper salt decomposed with H_2S . S-Methylcysteine is apt to contain traces of S—S compounds, as indicated by a positive cyanide-nitroprusside reaction. A preparation free from disulfides melted at 248° (uncorrected) and contained 10.4 per cent (10.37 calculated) of nitrogen and 24.2 per cent (23.7 calculated) of sulfur¹ (Pregl) and had an optical rotation of $[\alpha]_D^{20} = -23^\circ$.

γ, γ' -Dithiodibutyric acid was prepared as described by Gabriel (6), except that the condensation of KSCN with γ -chlorobutyronitrile was carried out for 24 hours instead of 2 hours. The compound melted at 106 – 107° and contained 26.6 per cent of S (calculated 26.9) by the Benedict method.

γ -Thiobutyric acid was prepared by reduction with zinc in N HCl at 50° for 1 hour. The acid solution was repeatedly extracted with ethyl acetate. The ethyl acetate solution was extracted with dilute NaOH and a solution of the sodium salt obtained, which was fed to the patient. The concentration of the sodium salt in this solution was determined by the Okuda method (7). The free thiol acid is easily reoxidized to the disulfide and was not obtained in crystalline form.

Metabolic Observations and Results—It can be seen that the excretion of the various urinary constituents by the cystinuric patient (A; Table I) and by the normal individual (N; Table II) was constant during the control periods. Throughout the experiments there was little change in the volume, pH, and specific gravity of the urine and in the excretion of ammonia and undetermined nitrogen, values for which were therefore omitted from Tables I and II. Tests for sulphydryl compounds in the urine were always negative. Cystine was determined by the Sullivan, Lugg-Sullivan, Folin-Marenzi, Folin photometric methods (*cf.* (2–4)), and also by the modification of the last procedure for homocystine (3, 4). In Table I (cystinuric) are reported only the cystine values obtained by the Lugg-Sullivan and Folin photometric

¹ It should be noted that the Benedict method gives low results for sulfur with S-methylcysteine. Since the total S determinations in the urine were carried out by this method, the values for total S and undetermined neutral S given in Tables I to III are somewhat low if an appreciable part of the undetermined neutral S consisted of S-methylcysteine.

TABLE I
Metabolic Observations on Cystinuric Patient A

Period No.	Date	Substance fed	Urine									
			Nitrogen		Creatinine		Sulfur				Cystine	
			Total	Urea	Formed	Total	Total	Inorganic SO ₄	Ethereal SO ₄	Neutral		Photometric
			gm.	gm.	gm.	gm.	gm.	gm.	gm.	Cystine	Undetermined	gm.
	1935		gm.									gm.
A33*	July 12-15		1 8	7 8	1 45	1 55	0 67	0 34	0 03	0 30	0 22	0 83
A34	" 16	S-Methylcysteine	1 8	7 5	1 37	1 54	0 77	0 30	0 02	0 39	0 21	0 80
	" 17		1 8	8 3	1 38	1 53	0 78	0 33	0 02	0 43	0 23	0 84
	" 18		3 6	7 7	1 45	1 57	0 87	0 35	0 02	0 50	0 23	0 87
A34a	" 19			7 6	1 41	1 61	0 86	0 37	0 03	0 46	0 23	0 84
	" 20			7 0	1 35	1 50	0 75	0 33	0 03	0 39	0 21	0 80
	" 21			7 6	1 41	1 57	0 76	0 38	0 02	0 36	0 22	0 82
	" 22			7 2	1 35	1 59	0 74	0 36	0 03	0 35	0 22	0 81
A35*	" 23			7 6	1 36	1 48	0 69	0 33	0 03	0 33	0 22	0 81
A39*	Aug. 6-7			7 2	1 32	1 47	0 65	0 31	0 04	0 31	0 23	0 87
A40	" 8		1 7	8 0	1 46	1 63	0 90	0 37	0 02	0 50	0 24	0 92
A40a	" 9	γ-Thiobutyric acid		7 6	1 36	1 47	0 71	0 36	0 03	0 32	0 21	0 80
A41*	" 10-11			7 5	1 27	1 47	0 67	0 32	0 03	0 32	0 22	0 81
A42	" 12	γ, γ'-Dithiodibutyric acid	3 0	8 7	1 44	1 52	1 39	0 44	0 03	0 92	0 24	0 96
A43*	" 13-14			8 2	1 32	1 41	0 70	0 35	0 04	0 31	0 22	0 83

* Average per day.

methods, while in Table II (normal) those obtained by the Folin-Marenzi and photometric methods are given. It should be noted that, as a result of the greater specificity of the photometric method, the "cystine" values obtained by this method in the normal are lower than the values given by the Folin-Marenzi method. Cystine estimations by the Sullivan procedures were always negative in the normal individual (with the quantities of urine employed). The presence of γ,γ' -dithiodibutyric acid has little influence on color formation in the Sullivan and Lugg-Sullivan

TABLE II
Metabolic Observations on Normal Human Being, Subject N

Period No.	Date	S-methyl-cysteine fed	Urine							
			Total N	Pre-formed creatinine	Sulfur				S-S groups calculated as "cystine"	
					Total	Inorganic-SO ₄	Ethereal SO ₄	Neutral	Folin-Marenzi	Folin photometric
	1935	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
N1*	Oct. 16-18		11.1	1.60	0.87	0.73	0.03	0.11	0.13	0.06
N2	" 19	1.8	11.8	1.60	1.07	0.88	0.02	0.17	0.10	0.07
	" 20	1.8	13.4	1.60	1.47	1.13	0.03	0.31	0.12	0.09
	" 21	3.6	11.5	1.56	1.35	1.03	0.02	0.30	0.13	0.07
N2a	" 22		11.6	1.60	1.14	0.88	0.05	0.21	0.11	0.07
	" 23		11.9	1.59	1.00	0.80	0.03	0.17	0.13	0.07
	" 24		11.7	1.48	1.01	0.83	0.03	0.15	0.15	0.09

* Average per day.

methods, in contrast to the marked inhibition we observed with dithiodiglycolic acid.

Both during the control and experimental periods there were only minor variations in the excretion of preformed creatinine, indicating conditions satisfactory for metabolic experimentation.

A summary and an analysis of the data are given in Table III. The method of calculation of the extra sulfur and that of its partition were described previously (1, 2).

S-Methylcysteine (Periods A34 and A34a and N2 and N2a)—The same amount (1.72 gm. of sulfur) was given to both the normal and the cystinuric individual. The substance proved to be non-toxic in the human, as indicated by the relative constancy of the

nitrogen excretion (Tables I and II) and by the reactions of the subjects. Du Vigneaud, Loring, and Craft (8) observed no apparent toxic effects with this compound in experiments with rabbits, while Pirie (9) reported that it was toxic to dogs. It should be noted from Tables I and II that following the administration of S-methylcysteine the rate of excretion of the extra sulfur was very slow, since the total sulfur did not return to its basal level for 4 days (Table I) and 3 days (Table II) respectively. This prolonged excretion was in the case of the cystinuric mainly extra neutral sulfur, while in the normal individual it was mostly accounted for by extra inorganic sulfate. The results obtained by the different cystine methods indicated that none of the extra neutral sulfur excreted in Periods A34 and A34a and Periods N2 and N2a was in the form of a disulfide. It is possible that the extra neutral sulfur excreted in these experiments is in part or in whole unchanged S-methylcysteine.

Table III shows that only 48 per cent of the sulfur of S-methylcysteine was excreted as extra sulfur by the cystinuric,¹ while all of the sulfur (106 per cent) was accounted for in the normal.¹ In the cystinuric, inorganic sulfate comprised 14 per cent and undetermined neutral sulfur 86 per cent of the extra S; there was no extra cystine. In the normal, the amount of oxidation was much greater, for 64 per cent of the extra S was sulfate and only 36 per cent neutral S.

γ -Thiobutyric Acid (Periods A40 and A40a)—Only a small amount of this substance (1.7 gm., equivalent to 0.45 gm. of S) was fed to the cystinuric patient, because we anticipated that it might be somewhat toxic, being a homologue of thioglycolic acid (for toxicity of this substance *cf.* (10)). An indication of the toxicity of thiobutyric acid can be found in the rise in the nitrogen excretion and perhaps in the somewhat higher cystine values in Periods A40 and A40a. It should be noted that the thiol compound was excreted to a considerable extent as a disulfide, presumably γ, γ' -dithiodibutyric acid. On account of the toxicity of the compound some of the extra sulfur excreted in this experiment is possibly derived from the tissues (for discussion *cf.* (10)), making the evaluation of the data by our usual method somewhat less significant. These figures were therefore omitted from Table III, but the calculations indicate that not more than 64 per cent of the

sulfur of the thiol compound was excreted as extra sulfur, of which about 30 per cent was inorganic sulfate S and 70 per cent neutral S (presumably the corresponding disulfide).

γ, γ' -Dithiodibutyric Acid (Periods A42 and A42a)—When 3 gm. (0.81 gm. of S) of this compound were fed to the cystinuric patient, the rate of excretion of the extra sulfur was very rapid, since the total S returned to the basal level 24 hours following the administration (see Table I). The disulfide, like its thiol analogue, was toxic, which was shown by a rise in the excretion of total nitrogen and of cystine. The evaluation of the data was, therefore, omitted from Table III, but the calculations indicated that about 75 per cent of the sulfur of the disulfide was excreted as extra sulfur, of which about 12 per cent was inorganic sulfate S and 88 per cent

TABLE III
Extra S and Its Partition in Experimental Periods

Subject	Period No.	S- methyl- cysteine fed	Amount of extra S				Extra S excreted as			
			Fed		Excreted		Inorganic SO ₄ -S		Undeter- mined neutral S	
			gm.	gm.	gm.	per cent	gm.	per cent extra S	gm.	per cent extra S
A Cystinuric	A34-A34a	7 2	1 72	0 83	48	0 12	14	0 71	86	
N. Normal	N2-N2a	7 2	1 72	1 82	106	1 17	64	0 65	36	

neutral S. It is possible that most, if not all, of the extra neutral sulfur excreted in this experiment was the unchanged disulfide; at least the values obtained by the various cystine methods are not contrary to such an assumption; this will be discussed in detail in a paper by Kassell and Brand on the Folin photometric method.

The lack of comparable data on the normal human being makes it difficult to decide whether or not the cystinuric (patient A) has a decreased ability to handle γ -thiobutyric acid and its disulfide analogue. It should be pointed out, however, that after feeding thioglycolic acid to rabbits (10), the partition of the extra sulfur (50 per cent of inorganic sulfate and of neutral S) was somewhat different from that observed after the feeding of γ -thiobutyric acid to the cystinuric individual (patient A).

DISCUSSION

S-Methylcysteine does not support the growth of rats on a cystine-deficient diet (8, 11, 12), although its sulfur is readily oxidized by rabbits (8). This was taken to indicate that demethylation is not the first step in the catabolism of this compound (3, 8). In agreement with this contention we find that when S-methylcysteine (and also S-ethylcysteine) was shaken in the Warburg apparatus with kidney slices no demethylation took place (*cf.* (13)), since the nitroprusside test remained negative. The observations reported in this paper on the metabolism of S-methylcysteine in human beings are in harmony with the results mentioned above. If demethylation with the formation of cysteine had taken place, the cystinuric patient should have excreted extra cystine in the experimental periods, since it has been found that cysteine administration raises the cystine output in cystinuria (2, 14). The oxidation of the sulfur of S-methylcysteine by the normal human being (subject N) was very similar to that by rabbits (8). In the normal human (subject N) inorganic sulfate accounted for 64 per cent of the extra S in contrast to only 14 per cent in the cystinuric. The ability of the cystinuric patient (A) to oxidize the sulfur of S-methylcysteine was, therefore, definitely decreased. It should be noted, however, that patient A excreted larger amounts of extra cystine following the administration of methionine and cysteine than other cystinuric individuals, indicating perhaps that the metabolic error is particularly marked in this patient.

The rise in the output of undetermined neutral sulfur following S-methylcysteine was presumably due to the excretion of the unchanged compound; at least no evidence to the contrary was obtained. Thus the metabolic behavior of S-methylcysteine is different from that of S-carboxymethylcysteine, since the latter substance was recently found to give rise to a mixed disulfide in the urine of both normals and cystinurics (15).

The slow excretion of the sulfur of S-methylcysteine was noted, but no explanation is offered for this observation.

Both γ -thiobutyric acid and γ, γ' -dithiodibutyric acid fail to support the growth of rats on a cystine-deficient diet (12) and do not yield extra cystine in the cystinuric. With thiobutyric acid it was noted as previously with cysteine, homocysteine, and glutathione (2) that sulfhydryl compounds are excreted as disulfides,

demonstrating again the possible rôle of the kidney in the oxidation-reduction mechanisms of S—S and —SH systems.

SUMMARY

1. S-Methylcysteine, γ -thiobutyric acid, and γ,γ' -dithiodibutyric acid do not yield extra cystine in cystinuria.

2. The sulfur of S-methylcysteine was oxidized by a cystinuric patient to a markedly less extent than by a normal human being.

3. The metabolic behavior of S-methylcysteine is different from that of S-carboxymethylcysteine.

4. γ -Thiobutyric acid was partially oxidized and partially excreted as an S—S compound, presumably the corresponding disulfide.

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STUDIES ON THE PRODUCTION OF TAUROCHOLIC ACID IN THE DOG*

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The theory stated in most text-books concerning the formation of taurine by the animal organism is based chiefly on the work of von Bergmann and of Whipple and his associates. On feeding cystine to bile fistula dogs von Bergmann (1) obtained no rise in the output of taurocholic acid, which he measured as alcohol-soluble sulfur. Giving cholic acid to such animals was followed by larger excretions of the conjugated bile acid, but continued feeding of cholic acid did not maintain these higher values. When cholic acid was administered with cystine, the dogs showed a marked production of taurocholic acid. Foster, Hooper, and Whipple (2) gave cholic acid to well fed bile fistula dogs and observed that production of taurocholic acid was augmented, but cholic acid did not cause a rise of the bile salt elimination immediately after the dogs had been fasting. These workers also found greater amounts of taurocholic acid after giving cystine and cholic acid together. Cystine alone failed to increase the taurocholic acid excretion. Taurocholic acid was calculated from the hydrolyzable amino nitrogen of the alcohol-soluble fraction of the bile. The experiments cited have been interpreted to indicate that the body can produce enough taurine for conjugation with all the cholic acid which is ordinarily available. Repeated feedings of cholic acid or periods of fasting may, however, deplete the body of its supply of taurine. A supply of cholic acid is the limiting factor in the synthesis of taurocholic acid. Cystine has been looked upon as the precursor of taurine.

* A preliminary report of these studies was given before the Thirty-first annual meeting of the American Society of Biological Chemists at Memphis, April 24, 1937.

Since the work cited above was completed, two new sulfur-containing amino acids, methionine and homocystine, have been discovered. Each of these compounds has been found capable of supporting growth on a cystine-deficient diet (3). It seemed of interest, therefore, to investigate the question as to whether either of these amino acids could give rise to the sulfur moiety of taurocholic acid.

EXPERIMENTAL

The general plan of these experiments was to give dogs cholic acid for a few days to deplete the liver of its store of taurine. A sulfur-containing amino acid was then fed with the cholic acid and its effect on taurocholic acid excretion measured.

Bile fistula dogs were prepared according to the technique of Rous and McMaster (4), except that the common duct was doubly ligated and cut, and the drainage tube was inserted in the cystic duct on removal of the gallbladder. This permitted the use of larger cannulas and diminished the danger of blockage. Furthermore, it seemed a sounder surgical procedure to allow the drainage tube to lie in the gallbladder fossa rather than across the pancreas and duodenum. When the animals had recovered from the operation they were put on a fasting régime and given 6.7 milli-equivalents (2.8 gm.) of cholic acid by capsule daily. On experimental days amino acids were fed in doses measured to contain 6.7 milli-equivalents each of sulfur; *i.e.*, 0.5 molecule of cystine and homocystine or 1 molecule of methionine was given for each molecule of cholic acid. The *d* and *l* forms of both methionine and homocystine (5) support growth, so it was not deemed necessary to feed double quantities of these compounds. The bile was collected at 24 hour intervals, just before the capsules were given, and the urine was collected by catheterization.

The bile was analyzed for taurocholic acid by the method of Foster and Hooper (6) with the following modification: the hydrolysis was carried out by heating 2 hours in an autoclave at 15 pounds pressure. Control experiments gave duplicate values with 24 hour hydrolyses in a steam bath. Several samples of bile were analyzed gravimetrically, following an alkaline oxidation of the alcohol-soluble sulfur fraction, and in no case was evidence for the presence of glycocholic acid obtained. This result does not

correspond with those of Josephson and Jungner (7) but is in accord with what others have found. In fact, the taurocholic acid as calculated from the alcohol-soluble sulfur was frequently greater than that calculated from the hydrolyzable amino nitrogen.

Urinary sulfur partitions were made according to Denis (8). On the days after methionine was given the total sulfur values were checked by alkaline oxidations, and duplicate values were invariably obtained. Incomplete oxidations of methionine by the Denis reagent as reported by Painter and Franke (9) were the cause of making these check determinations. Our results would indicate that no unchanged methionine was excreted in the urine. Nitrogen was determined by the Kjeldahl method, and disulfide compounds were estimated according to Virtue and Lewis (10). While the Virtue-Lewis method as applied to dog urine has an accuracy probably not greater than 90 per cent, it measures quantities small enough to render it most valuable for the purpose desired.

The c.p. cholic acid used was purchased from Riedel-de Haen. The cystine was the natural isomer, isolated from human hair. The alanine, homocystine, and methionine were synthetic products.

Results

The results shown for the first 6 days in Table I illustrate the fact that administration of cholic acid produces a rise in the level of taurocholic acid output, which falls off when cholic acid is given on succeeding days. This falling off may also be seen in Tables II, III, and IV. Tables I through IV give examples of the extra production of taurocholic acid when both cholic acid and cystine are fed. The results are in accord with those of von Bergmann and of Whipple. Cystine and cholic acid were fed five times in our series, and similar effects were seen in each case.

Administration of methionine was also followed by larger excretions of taurocholic acid, as may be seen in Tables I, II, IV, and V. To make sure that the material conjugated with cholic acid and determined by its hydrolyzable amino nitrogen was actually taurine and not a 3-carbon homologue, isolation experiments were performed. The material obtained melted at 313° (uncorrected) (see White and Fishman (11)) and showed no

depression of the melting point when mixed with synthetic taurine. Methionine was given with cholic acid seven times, and each time gave rise to extra taurocholic acid.

To determine whether the bile salt formation might be due to simply an amino acid stimulation, *dl*-alanine was given twice with cholic acid. Neither experiment caused extra taurocholic acid excretion. The results of one of these feedings are shown in Table

TABLE I

Effect of Cystine and Methionine on Production of Taurocholic Acid

Dog 16; fasting throughout experiment. Weight 8.1 kilos at start, 6.4 kilos at finish.

Day of experiment	Total N	Total S	Sulfate S	Organic S	Disulfide S	Taurocholic acid
	gm.	mg.	mg.	mg.	mg.	mg.
1	2.68	209	162	47	3	882
2	1.99	146	110	36	2	613
3	2.19	163	135	28	3	1253*
4	2.46	151	126	25	2	917*
5	2.43	155	121	34	3	505*
6	2.79	157	131	26	3	511*
7	2.83	308	279	29	2	1213†
8	3.13	195	150	45	3	122*
9	3.25	305	262	43	4	1368‡
10	2.28	119	97	22	1	1384*
11	2.52	142	116	26	1	600*

* 6.7 milli-equivalents of cholic acid (2.8 gm.) fed.

† 6.7 milli-equivalents of cholic acid + 6.7 milli-equivalents of sulfur (213 mg.) as cystine (0.8 gm.) fed.

‡ 6.7 milli-equivalents of cholic acid + 6.7 milli-equivalents of sulfur as methionine (1.0 gm.) fed.

II. The amount of *dl*-alanine used in each case was equivalent to twice the cholic acid used, because of possible non-utilization of the inactive form.

Table III shows that no extra production of taurocholic acid resulted from administration of homocystine with cholic acid. Table IV, however, indicates the possibility of such a bile acid formation, since the level of bile acid output was somewhat raised for 3 days following the feeding of homocystine. It should be noted here that the basic level rose on the 9th day of the experi-

TABLE II

Effect of Cystine, Alanine, and Methionine on Production of Taurocholic Acid

Dog 26; fasting throughout. 2.8 gm. = 6.7 milli-equivalents of cholic acid fed daily. Weight at start 15.4 kilos, at finish 12.7 kilos.

Day of experiment	Total N	Total S	Sulfate S	Organic S	Disulfide S	Taurocholic acid
	gm.	mg.	mg.	mg.	mg.	mg.
1	5.15	243	186	57	3	2184
2	4 11	166	126	40	1	1110
3	3 91	149	108	41	1	1239
4	3 79	284	247	37	2	1895*
5	4 86	220	134	86	0	1069
6	3 51	123	81	42	1	1382
7	2 27	141	105	36	1	715†
8	2 71	326	280	46	2	2492‡
9	2 50	194	157	37	2	1181

* 6.7 milli-equivalents of cholic acid (2.8 gm.) + 6.7 milli-equivalents of sulfur (213 mg.) as cystine (0.8 gm.) fed.

† 6.7 milli-equivalents of cholic acid + 13.4 milli-equivalents of *dl*-alanine (1.2 gm.) fed.

‡ 6.7 milli-equivalents of cholic acid + 6.7 milli-equivalents of sulfur as methionine (1.0 gm.) fed.

TABLE III

Effect of Homocystine and Cystine on Production of Taurocholic Acid

Dog 21; fasting. 6.7 milli-equivalents of cholic acid (2.8 gm.) given orally daily. Weight at start 10.7 kilos, at finish 7.7 kilos.

Day of experiment	Total N	Total S	Sulfate S	Organic S	Disulfide S	Taurocholic acid
	gm	mg.	mg	mg.	mg	mg.
1	3 68	154	119	35	2	949
2	2 12	126	96	30	1	312
3	2 25	123	95	28	1	244
4	1 37	60	41	19	1	649
5	2 93	244	184	60	7	785*
6	2 46	112	80	32	1	400
7	2 44	108	78	30	1	506
8	2 04	103	75	28	1	770
9	1.82	217	183	34	1	1227†

* 6.7 milli-equivalents of sulfur (213 mg.) as homocystine (0.9 gm.) fed with the cholic acid.

† 6.7 milli-equivalents of sulfur as cystine (0.8 gm.) fed with the cholic acid.

mental period, which was the day before the homocystine was given. In view of the doubtful nature of this rise, further experiments were performed to ascertain the true status of homocystine as a precursor of taurine. Table V is one example of results from three other dogs. These three experiments are quite clear cut in

TABLE IV

Effect of Methionine, Homocystine, and Cystine on Production of Taurocholic Acid

Dog. 20; fasting throughout experiment. Weight 12.3 kilos at start, 7.7 kilos at finish.

Day of experiment	Total N	Total S	Sulfate S	Organic S	Disulfide S	Taurocholic acid
	gm.	mg.	mg.	mg.	mg.	mg.
1	Urine lost					1004
2	5.25	213	157	56	8	459
3	3.51	169	125	44	4	625*
4	3.61	176	129	47	3	712*
5	3.31	155	112	43	3	753*
6	4.12	292	241	51	4	1381†
7	4.71	246	202	44	2	958*
8	Urine lost					525*
9	3.65	175	141	34	3	907*
10	4.12	291	239	52	7	1219‡
11	3.91	227	187	40	2	1202*
12	3.99	198	161	37	6	1429*
13	3.97	169	134	35	5	981*
14	3.66	164	130	34	3	971*
15	4.67	390	343	47	7	1933§

* 6.7 milli-equivalents of cholic acid (2.8 gm.) fed.

† 6.7 milli-equivalents of cholic acid + 6.7 milli-equivalents of sulfur (213 mg.) as methionine (1.0 gm.) fed.

‡ 6.7 milli-equivalents of cholic acid + 6.7 milli-equivalents of sulfur as homocystine (0.9 gm.) fed.

§ 6.7 milli-equivalents of cholic acid + 6.7 milli-equivalents of sulfur as cystine (0.8 gm.) fed.

showing that homocystine when given with cholic acid is unable to cause an increased production of taurocholic acid. At the same time the urinary sulfur figures (Tables III, IV, and V) indicate that the sulfur of homocystine is oxidized by the dog to approximately the same extent as the sulfur of cystine or meth-

ionine. Du Vigneaud, Loring, and Craft have shown this to be true for the rabbit (12).

Small amounts of some disulfide material which was not cystine, as shown by negative Sullivan tests, were excreted consistently on the day following the administration of homocystine (Tables III to V). This could have been unchanged homocystine. No such disulfide material was found in the urine after feeding methionine, which is what Andrews and Randall (13) found with dogs. Virtue and Lewis (14) have previously reported the occurrence of a disulfide reaction after giving methionine to rabbits, but the dose of methionine in that case was nearly 5 times as much per kilo of body weight.

TABLE V

Effect of Homocystine and Methionine on Production of Taurocholic Acid

Dog 32; fasting throughout. 2.8 gm. = 6.7 milli-equivalents of cholic acid fed daily. Weight at start 9.3 kilos, at finish 7.7 kilos.

Day of experiment	Total N	Total S	Sulfate S	Organic S	Disulfide S	Taurocholic acid
	gm.	mg.	mg.	mg.	mg.	mg.
1	3.60	219	172	47	3	1688
2	5.40	270	220	50	4	1352
3	6.51	418	348	70	12	1573*
4	5.12	247	198	49	2	1417
5	4.82	348	287	61	5	2771†
6	6.53	203	150	53	1	1814

* 6.7 milli-equivalents of sulfur (213 mg.) as homocystine (0.9 gm.) fed.

† 6.7 milli-equivalents of sulfur as methionine (1.0 gm.) fed.

DISCUSSION

The ideas advanced by both von Bergmann and Whipple to explain the formation of taurocholic acid from cholic acid and cystine are well supported by our results. Interference of dietary factors has been eliminated in these experiments by the use of fasting animals. In addition, these results show that the administration of methionine with cholic acid is followed by the excretion of extra bile salts, of which the sulfur moiety is taurine. We have no evidence as to whether methionine is changed directly to taurine or whether it may replace some substance in the tissues such as cystine, which might then be changed to taurine.

If we assume that cystine or methionine is changed directly to taurine, it is of interest to estimate what proportion of the sulfur fed is excreted by known pathways. In our experiments each administration of a sulfur-containing amino acid furnished the animal 213 mg. of sulfur. Approximately 150 mg. of this were excreted as extra sulfur in the urine. While the normal variations of bile acid output are rather large, it appears that approximately 700 mg. of extra taurocholic acid were excreted in the bile following each feeding with cholic acid plus cystine or methionine. This corresponds to 42 mg. of sulfur. These two paths of excretion therefore account for about 192 mg., or 90 per cent of the ingested sulfur. Part of the remaining 10 per cent is excreted in the bile in some form other than taurocholic acid.

The suggestion that one of the first steps in the catabolism of methionine is demethylation to form homocysteine (14) arose from finding extra disulfide material in the urine after the administration of methionine. It is quite possible that homocysteine is a normal intermediate in the catabolism of methionine and that only small amounts of homocysteine are formed by oxidation, which would account for the disulfide excretion observed. If homocysteine is a normal intermediate in the breakdown of methionine, one should expect homocysteine to cause taurine production even more readily than does methionine. Our results indicate that homocysteine does not readily lead to the formation of taurine, even though the sulfur of homocysteine is oxidized as easily as that of methionine. There is a possibility which we hope to investigate which might throw light on the situation; namely, that homocysteine might give rise to the production of taurocholic acid. Evidence has already been produced that homocysteine and homocysteine may have different paths of metabolism in the cystinuric individual (15).

SUMMARY

1. After administration of cholic acid to fasting bile fistula dogs for several days, the administration of either cystine or methionine with the cholic acid resulted in an increased output of taurocholic acid in the bile.

2. When homocysteine was given with the cholic acid under these conditions, the level of taurocholic acid excretion was not raised, except in one doubtful experiment.

3. The dog excretes most of the sulfur of homocystine in the urine as inorganic sulfate.

The authors wish to acknowledge their indebtedness to Dr. A. G. Eaton for his cooperation in making this work possible. We also wish to thank Dr. Vincent du Vigneaud for a generous gift of homocystine.

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THE RÔLE OF SPECIFICITY IN THE ENZYMATIC SYNTHESIS OF PROTEINS

SYNTHESES WITH INTRACELLULAR ENZYMES

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The enzymes which participate in the natural process of protein formation from simpler structural units should be capable of synthesizing peptide linkages. In performing the synthesis of peptide bonds, these enzymes should also be capable of making a precise selection among the available structural units, so that the complicated pattern of the individual protein molecule may be obtained. The experiments reported in the present paper are devoted to a study of the specificity of enzymes that synthesize peptide linkages.

The question whether proteolytic enzymes may have synthetic activity has already been studied by numerous investigators.¹ Danilewski (3) in 1886 reported the first enzymatic synthesis of protein-like material, and Sawjalow (4) in 1901 suggested the name "plastein" for this material. Wasteneys and Borsook (1) in their well known series of papers found the synthetic action of pepsin and trypsin to have an optimum at pH 4 and to be favored by a rise of the reaction temperature and of the substrate concentration. With respect to intracellular proteinases, Voegtlin and his collaborators (5) regard the following conditions as favoring synthetic processes: (a) a relatively high oxygen tension; (b) a hydrogen ion concentration not far removed from neutrality; (c) a relatively high initial concentration of —SH groups which can give rise to a relatively high concentration of disulfides; (d) a sufficient concentration of suitable protein split-products.

¹ For more complete bibliographies, see Wasteneys and Borsook (1) and Oppenheimer (2).

Erepsin was shown by von Euler and Sjöman (6) to produce a definite decrease of alkalinity in water-glycerol solutions of amino acids; this has been interpreted as demonstrating the occurrence of peptide synthesis.

Proteolytic enzymes are, then, supposed to effect synthesis as well as hydrolysis. It is assumed that the general physicochemical conditions such as pH, concentration, oxygen tension, and temperature determine whether hydrolysis or synthesis shall predominate.

In order to approach the problem mentioned above of enzymatic specificity, several simple enzymatic syntheses have been performed.

When a dilute solution containing 4.2 per cent of carbobenzoxyglycine and 3.7 per cent of aniline is incubated with activated papain at 40° and pH 4.6, carbobenzoxyglycine anilide is formed. Under our experimental conditions, the anilide begins to crystallize after about 1 hour; after a few days, 80 per cent of the theoretical amount may be isolated. An anilide can be obtained from hippuric acid under similar conditions.

The formation of the anilides does not take place when the papain preparation has been freed of natural activators and no other activator has been added. Cysteine, glutathione, and HCN have been found to activate papain for the syntheses of the anilides, while cystine does not activate the enzyme.

The optimum pH range of the enzymatic formation of carbobenzoxyglycine anilide has been found to be close to 4.6 (see Fig. 1). It will be noted that the conditions of pH, concentration, temperature, and activation which permit the enzymatic synthesis of the above anilides are identical with those conditions usually employed in proteolytic experiments with papain. Thus it becomes apparent that papain may, under identical general conditions, perform hydrolyses as well as syntheses. Whether, in a given case, hydrolysis or synthesis prevails depends upon the nature of the substrate or substrates, and rather small structural differences may influence the course of the reaction. For example, hippurylamide is completely hydrolyzed to hippuric acid and ammonia (7) under the same conditions that effect the synthesis of hippurylanilide from hippuric acid and aniline.

On the basis of the foregoing findings it was to be expected that

on treating hippurylamide with aniline in the presence of activated papain, hippurylanilide should be formed. Moreover, the experiments presented in Fig. 2 show that the anilide is formed from hippurylamide at a rate which exceeds the rate of anilide formation from hippuric acid. It appears, therefore, that the transformation of the amide into the anilide does not proceed through the intermediate stage of hippuric acid, but that the NH_2 group in the molecule of hippurylamide is directly replaced by the NHC_6H_5 group.

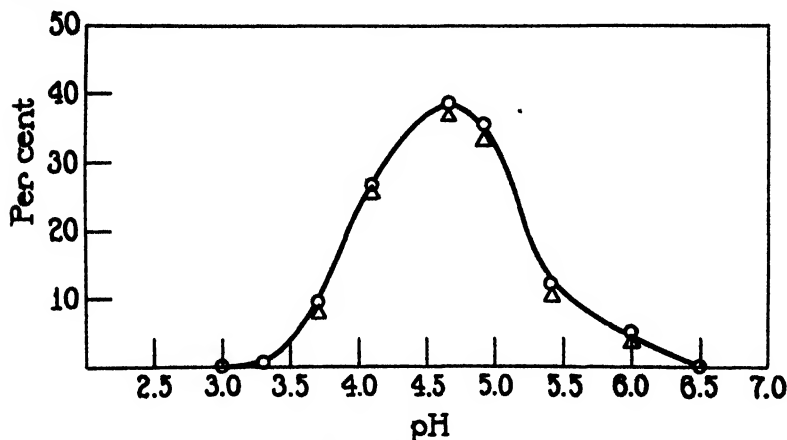


FIG. 1. pH dependence of synthesis of carbobensoxyglycylanilide by papain-cysteine. O = amount of anilide isolated after 26 hours, expressed in per cent of theoretical maximum; Δ = percentage of anilide formed as determined by titration after 23 hours. Concentration of carbobensoxyglycine, 2.1 per cent; aniline, 1.85 per cent. The pH values were determined by means of the glass electrode.

The fact that an enzyme is able to transform one amide bond into another leads to the expectation that similar transformations of peptide bonds may play a rôle in the biological synthesis of individual proteins. On the other hand, one must consider the possibility that transformations of a similar kind may, in some cases, take place during the enzymatic hydrolysis of proteins and protein split-products *in vitro*. In such a case, combinations of amino acids which did not exist in the original material may be found in the enzymatic digest.

It has been shown (8) that papain consists of two partial en-

zymes, called Papain I and Papain II, which were found to differ in specificity. The syntheses described in this paper are due to the action of Papain I. This may be demonstrated on the basis of the following facts. The binary papain or holopapain produces its two partial enzymes Papain I and Papain II by a process of dissociation. The dissociation can be achieved by means of phenylhydrazine, resulting in a proteolytically inactive compound Papain I-phenylhydrazine and an active Papain II. When holopapain is treated with phenylhydrazine in the presence of HCN, the same result is obtained: Papain II is active, whereas Papain I

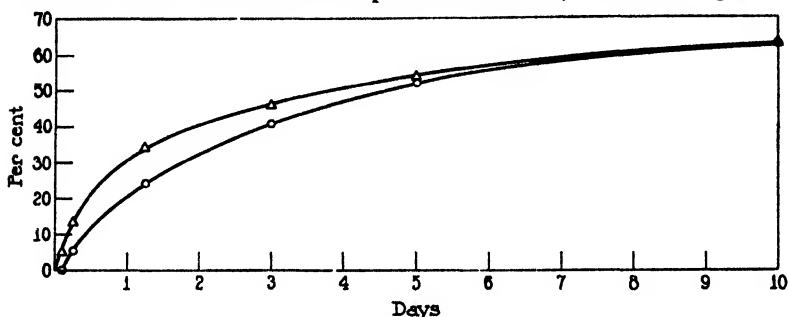


FIG. 2. Rate of formation of hippurylanilide from hippuric acid and from hippurylamide. ○ = amount of anilide isolated from papain action on hippuric acid and aniline; △ = amount of anilide isolated from papain action on hippurylamide and aniline. The extent of synthesis is expressed in percentage of the theoretical maximum. Concentration of hippuric acid or hippurylamide, 1.8 per cent; aniline, 2 per cent. In the case of hippurylamide, an equivalent amount of acetic acid was added to neutralize the ammonia which formed. The pH was checked electrometrically; it was 4.70 for the hippuric acid experiment and 4.77 for the hippurylamide experiment, and did not change in the course of the reaction.

is inactivated. HCN is not capable of liberating Papain I from its combination with phenylhydrazine. When, however, holopapain is treated with phenylhydrazine in the presence of cysteine, Papain I retains its activity to an appreciable degree, while Papain II is fully active, as in the other cases; cysteine is thus capable of splitting the combination Papain I-phenylhydrazine with the liberation of active Papain I (9). These effects of phenylhydrazine on papain are indicated in Table I.

Thus, the question whether an enzymatic process is due to Papain I or Papain II can easily be decided by comparing the

process in the presence of phenylhydrazine and HCN with its course in the presence of phenylhydrazine and cysteine. If the reaction under consideration proceeds in the presence of phenylhydrazine and cysteine but does not occur when HCN takes the place of cysteine, then the reaction may be attributed to Papain I.

In the application of this discriminative method to syntheses with carbobenzoxyglycine and papain, it was found that phenylhydrazine has a 2-fold capacity: in the presence of HCN it inhibits the enzyme, while in the presence of cysteine it not only fails to inhibit the enzyme but itself enters the synthetic reaction in forming carbobenzoxyglycine-phenylhydrazide. On the basis of these findings, the synthesis of this phenylhydrazide must be attributed to Papain I. Furthermore, the synthesis of carbobenzoxyglycine anilide from carbobenzoxyglycine and aniline by means of papain-HCN is almost completely inhibited by the

TABLE I
Activity of Papain in Presence of Phenylhydrazine, Hydrocyanic Acid, and Cysteine

Phenylhydrazine	Phenylhydrazine-HCN	Phenylhydrazine-cysteine
Papain I inactive " II active	Papain I inactive " II active	Papain I active " II "

addition of phenylhydrazine. Consequently, the synthesis of the anilide is due to Papain I.

It was possible to demonstrate, furthermore, that the synthesizing action of papain is not restricted to derivatives of glycine. Acetyl, benzoyl, and carbobenzoxy derivatives of alanine, leucine, and phenylalanine could be transformed into anilides or phenylhydrazides, usually with a high or even quantitative yield. With benzoylsarcosine, however, no synthesis of the anilide or of the phenylhydrazide could be achieved by means of papain-cysteine. Moreover, no synthesis could be effected when in place of the previously mentioned acylated amino acids the corresponding free amino acids were treated with papain-cysteine and aniline or phenylhydrazine. It seems probable, therefore, that as a synthesizing enzyme Papain I uses as its substrate the structural group $R \cdot CO-NH \cdot CHR' \cdot COOH$.

The antipodal specificity of Papain I as a synthesizing enzyme

was revealed when acyl derivatives of *dl*-alanine, *dl*-leucine, and *dl*-phenylalanine were incubated with aniline or phenylhydrazine and papain-cysteine. In all cases the enzyme confined its action strictly to the derivative of the natural *l*-amino acid, leaving the derivative of the *d*-amino acid unchanged. Thus, from a homogeneous preparation of benzoyl-*d*-leucine no anilide could be obtained under conditions in which benzoyl-*l*-leucine was transformed into its anilide with an almost quantitative yield.

It may be mentioned that syntheses of the kind described in this paper are not restricted to papain. Bromelin, the proteolytic enzyme of the pineapple, also synthesizes carbobenzoxyglycine anilide from carbobenzoxyglycine and aniline. Since papain and bromelin have been shown to differ in their specificity, it is not surprising that with bromelin much lower yields of the anilide are obtained than with papain.

Another example of a synthesis effected by bromelin is the condensation of benzoyl-*l*-leucine and aniline to benzoyl-*l*-leucine anilide. The synthesis is due to that partial enzyme of bromelin which has been called Bromelin I (9). When benzoyl-*dl*-leucine has been used as starting material, the anilide of benzoyl-*l*-leucine has been obtained. Bromelin I, therefore, shows antipodal specificity.

The syntheses of carbobenzoxyglycine anilide and benzoyl-*l*-leucine anilide could also be performed with the aid of pig liver cathepsin. Here the yields were still lower than those obtained with bromelin. It was found that cathepsin shows the same antipodal specificity as do papain and bromelin.

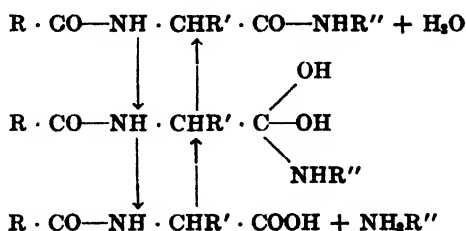
None of the many proteins which have been investigated up to the present time has been shown to contain a *d*-amino acid as a constituent. Even when a *d*-amino acid had been fed to growing animals, the proteins formed during the process of growth did not contain this *d*-amino acid (10). The complete absence of *d*-amino acids from proteins may be a consequence of the antipodal specificity of the enzymes that synthesize proteins. For example, when Papain I, Bromelin I, or cathepsin participates in the synthesis of a protein *in vivo*, it will admit only the *l* form of the asymmetric amino acids into the protein molecule under construction.

The antipodal specificity of papain, bromelin, and cathepsin as synthesizing enzymes can be explained with the aid of the

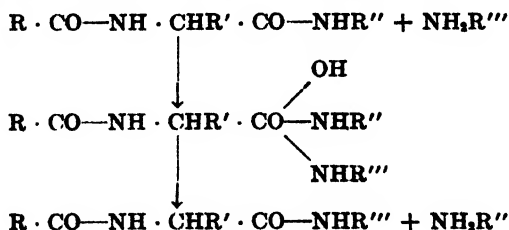
polyaffinity theory. A detailed discussion of the polyaffinity theory of enzyme action has been given in a previous paper on Papain I as a hydrolytic enzyme (11).

The antipodal specificity of Papain I can be utilized as a method to resolve *dl*-amino acids. As an example, the preparation of *d*-leucine may be described. When carbobenzoxy-*dl*-leucine is treated with papain-cysteine and aniline, the derivative of the *l* component is quantitatively transformed into the anilide. This, due to its very slight solubility in water, can be removed by filtration. The carbobenzoxy-*d*-leucine in the mother liquor after hydrogenation of the carbobenzoxy group yields *d*-leucine in excellent yield and purity. Since the *l* forms of amino acids are available from protein hydrolysates, it is a desirable fact that the specificity of papain, in combination with the carbobenzoxy method, offers in several cases an opportunity to isolate the *d* form.

There are at present three types of reaction known to be effected by intracellular enzymes: hydrolysis of peptide bonds, synthesis of peptide bonds, and replacement of one participant in a peptide bond by another. The three reaction types are represented by the following sets of formulas.



Scheme of a hydrolysis (arrows at the left side) and a synthesis (arrows at the right side) of peptide bonds, as effected by intracellular enzymes.



Scheme of a mutual replacement of participants in a peptide bond.

If several peptides are simultaneously in contact with an intracellular enzyme, the character of the respective residues R, R', R'', R''' in conjunction with the individual specificity of the enzyme involved will determine whether and to what extent hydrolysis, synthesis, or replacement may occur. In many cases, several types of reaction may be combined. An example of such a combination of several types of enzymic reactions will be described in a following paper.

The findings reported in this paper may influence the present concept of protein synthesis *in vivo*. It now becomes probable that the processes of protein synthesis and protein hydrolysis may occur *in vivo* under identical physicochemical conditions, and often simultaneously. Furthermore, protein synthesis *in vivo* must not necessarily proceed in such a way that one amino acid residue after another is attached to the rudimentary molecule of the protein under construction. One must consider also the alternative that the enzyme has at its disposal a number of protein fragments of different size and structure and subjects these fragments to a series of transformations until there is produced a protein pattern which is stable in the presence of the individual enzyme. Thus, the available protein fragments and the specificity of the enzyme together may determine the individual pattern of the synthesized protein.

We wish to acknowledge with thanks the very valuable technical assistance of Mr. Joseph L. Goldberg who performed the analyses reported in this paper.

EXPERIMENTAL

Papain Preparation—A papain preparation, obtained in Ceylon from the sap of *Carica papaya* by careful vacuum evaporation and kept in storage at a temperature of approximately 5°, was dissolved in water, bubbled for several hours with H₂S, and precipitated with alcohol. The treatment with H₂S and alcohol was repeated twice and the precipitate finally obtained was kept in a vacuum desiccator over P₂O₅.

General Procedure for Syntheses with Papain—90 mg. of purified papain were suspended in a mixture of 10 cc. of water and 10 cc. of citrate buffer (pH 5) and filtered after $\frac{1}{2}$ hour. Of this papain

solution, 10 cc. were mixed with 15 cc. of citrate buffer (pH 5). After addition of the substrates and of the activator, the solution was made up with water to 50 cc. and incubated in a tightly stoppered flask. The size of the flask was a little more than 50 cc.

The pH was tested in all experiments by means of the glass electrode.

Synthesis of Carbobenzoxylglycine Anilide with Papain—2.1 gm. of carbobenzoxylglycine, 1.85 gm. of aniline, and 50 mg. of cysteine hydrochloride were incubated with a papain-buffer solution at 40°, as previously described. After about 1 hour crystallization of the synthesized anilide began. After 4 days 1.75 gm. of anilide had separated out, corresponding to 61.5 per cent of the theory. With the double amount of papain and cysteine, a yield corresponding to 81 per cent of the theory was obtained.

Carbobenzoxylglycine anilide melts at 144°. It was recrystallized from dilute alcohol (50 per cent).

$C_{16}H_{16}O_2N_2$.	Calculated.	C 67.6, H 5.7, N 9.9
284.3	Found.	" 67.7, " 5.8, " 10.0

When papain-HCN was used instead of papain-cysteine, a yield of 46 per cent was obtained after 4 days. When 0.15 cc. of phenylhydrazine was added, no synthesis took place with papain-HCN.

Synthesis of Hippurylanilide with Papain—0.9 gm. of hippuric acid and 1 gm. of aniline were incubated at 40° with papain-cysteine. After 3 days 525 mg. of hippurylanilide separated out, corresponding to 43 per cent of the theory. M.p., 212.5°.² For analysis the anilide was dried at 100° *in vacuo*.

$C_{16}H_{14}O_2N_2$.	Calculated.	C 70.9, H 5.5, N 11.0
254.3	Found.	" 70.8, " 5.7, " 11.1

Transformation of Hippurylamide into Hippurylanilide with Papain—0.9 gm. of hippurylamide and 1 gm. of aniline were incubated with papain-cysteine at 40°. At the beginning 0.3 gm. of glacial acetic acid was added in order to avoid a shift of the pH due to the ammonia which was liberated during the reaction. 46

² Curtius (12) gives a melting point of 208.5° for this material.

per cent of the theoretical amount of hippurylanilide separated out after 3 days, 55 per cent after 5 days, and 64 per cent after 10 days. After the material was recrystallized from dilute alcohol and dried at 100°, the melting point was 212.5°.

$C_{15}H_{14}O_2N_2$.	Calculated.	C 70.9, H 5.5, N 11.0
254.3	Found.	" 70.8, " 5.4, " 11.1

Synthesis of Carbobenzoxyglycine Phenylhydrazide with Papain—1 gm. of carbobenzoxyglycine and 0.5 gm. of phenylhydrazine incubated with papain-cysteine at 40° yielded, after 24 hours, 58.5 per cent of the theoretical amount of carbobenzoxyglycine phenylhydrazide.

0.825 gm. of carbobenzoxyglycine and 0.41 gm. of phenylhydrazine yielded 67.5 per cent of the phenylhydrazide after 2 days. A corresponding experiment, in which the addition of cysteine was replaced by HCN, yielded only 0.5 per cent of phenylhydrazide after 2 days.

An experiment in which 0.5 gm. of carbobenzoxyglycine and 0.3 gm. of phenylhydrazine were incubated with papain-cysteine for 6 days yielded 84 per cent of the theoretical amount of phenylhydrazide.

After recrystallization from absolute alcohol, the phenylhydrazide melted at 144°.

$C_{15}H_{17}O_2N_2$.	Calculated.	C 64.2, H 5.7, N 14.0
299.3	Found.	" 64.0, " 5.9, " 14.3

Synthesis of Hippurylphenylhydrazide with Papain—0.45 gm. of hippuric acid and 0.3 gm. of phenylhydrazine incubated with papain-cysteine at 40° for 6 days separated out 84 per cent of the theoretical amount of phenylhydrazide. After the material was recrystallized from absolute alcohol the melting point was 184.5°.³

$C_{15}H_{15}O_2N_2$.	Calculated.	C 66.9, H 5.6, N 15.6
269.3	Found.	" 66.9, " 5.6, " 15.7

Synthesis of Benzoyl-L-Leucine Anilide with Papain—1.2 gm. of benzoyl-DL-leucine and 1 gm. of aniline were incubated with papain-cysteine at 40° with the addition of 6 cc. of 2 M sodium acetate solution. After only a few minutes the anilide began to

³ Curtius (12) gives a melting point of 182.5° for this substance.

precipitate out. After 20 hours its amount was 96 per cent of the theoretical value calculated for one antipode. 2 days later another 3 to 4 per cent had formed. Recrystallized from absolute alcohol, the material had a melting point of 213°.

$C_{11}H_{23}O_2N_2$. Calculated. C 73.5, H 7.1, N 9.0
 310.4 Found. " 73.6, " 7.2, " 9.2
 $[\alpha]_D^{25} = +9.0^\circ$ (5% in glacial acetic acid)

1.2 gm. of benzoyl-*l*-leucine yielded, under corresponding experimental conditions, 91.6 per cent and after 2 more days 7.9 per cent of the anilide which had a specific rotation of $+9.0^\circ$. 1.2 gm. of benzoyl-*d*-leucine yielded no anilide at all. If optically impure preparations containing small amounts of benzoyl-*l*-leucine were employed, corresponding amounts of benzoyl-*l*-leucine anilide were obtained.

Synthesis of Benzoyl-l-Phenylalanine Anilide with Papain—1.4 gm. of benzoyl-*l*-phenylalanine and 1.1 gm. of aniline were incubated at 40° with papain-cysteine with the addition of 3.6 cc. of *m* NaOH and 5.4 cc. of 2 *m* sodium acetate. After 5 days 66.5 per cent of the theoretical amount of anilide was obtained. After the material was recrystallized from alcohol, the melting point was 219–220°.

$C_{22}H_{21}O_2N_2$. Calculated. C 76.7, H 5.9, N 8.1
 344.4 Found. " 76.8, " 5.9, " 8.0
 $[\alpha]_D^{25} = +27.6^\circ$ (5% in pyridine)

An experiment with benzoyl-*dl*-phenylalanine yielded an anilide of the same melting point, the same composition, and $[\alpha]_D^{25} = +27.9^\circ$.

Synthesis of Acetyl-l-Phenylalanine Phenylhydrazide with Papain—4.2 gm. of acetyl-*l*-phenylalanine and 2.2 gm. of phenylhydrazine were incubated with papain-cysteine at 40° with the addition of 4.08 gm. of sodium acetate. After 11 days 33 per cent of the theoretical amount of phenylhydrazide had separated out. After recrystallization from dilute alcohol (33 per cent) the material had a melting point of 205°.

$C_{17}H_{19}O_2N_3$. Calculated. C 68.7, H 6.4, N 14.1
 297.3 Found. " 68.9, " 6.5, " 14.2
 $[\alpha]_D^{25} = -33.5^\circ$ (4.5% in pyridine)

With acetyl-*dl*-phenylalanine as a starting material, a phenylhydrazide showing $[\alpha]_D^{23} = -33.4^\circ$ was obtained. Yield after 2 days, 720 mg. When HCN replaced cysteine as an activator, 80 mg. of phenylhydrazide were obtained after 2 days.

Synthesis of Benzoyl-L-Alanine Anilide with Papain—1.9 gm. of benzoyl-*L*-alanine and 1 gm. of aniline, incubated at 40° with papain-cysteine, yielded 85 per cent of benzoyl-*L*-alanine anilide in the course of 4 days. After the material was recrystallized from dilute alcohol, the melting point was $175\text{--}176^\circ$.

$C_{16}H_{16}O_2N_2$.	Calculated.	C 71.6, H 6.0, N 10.4
268.3	Found.	" 71.8, " 6.2, " 10.5
$[\alpha]_D^{23} = -8.0^\circ$	(5% in glacial acetic acid)	

Synthesis of Benzoyl-L-Leucine Anilide with Bromelin—1.2 gm. of benzoyl-*dl*-leucine and 1 gm. of aniline were incubated at 40° with bromelin-cysteine under conditions analogous to those previously described for the syntheses with papain. After 4 days, 64 per cent of the theoretical amount calculated for one antipode separated out. M.p., 213° . $[\alpha]_D^{23} = +9.2^\circ$ (5 per cent in glacial acetic acid).

0.24 gm. of benzoyl-*L*-leucine and 0.2 gm. of aniline were incubated with bromelin-HCN in 10 cc. of solution. After 2 days, 28 mg. of anilide had formed, whereas with addition of phenylhydrazine (0.03 mm per cc.) the same experiment yielded only traces of the anilide.

Synthesis of Carbobenzoxyglycine Anilide with Bromelin—0.85 gm. of carbobenzoxyglycine and 0.75 gm. of aniline, incubated at 40° in a 20 cc. solution with bromelin-cysteine, after 4 days yielded 30 mg. of anilide. M.p., $144\text{--}145^\circ$. Mixed melting point, with an anilide of another source, 145° .

Synthesis of Benzoyl-L-Leucine Anilide with Cathepsin—0.48 gm. of benzoyl-*dl*-leucine and 0.4 gm. of aniline were incubated with cathepsin from pig liver in the presence of cysteine in 20 cc. of solution. After 4 days the precipitated crystals were filtered and recrystallized by solution in alcohol, filtration from accompanying amorphous substances, and precipitation with water. Yield, 6 mg. M.p., 210° . A mixture with benzoyl-*L*-leucine anilide with a melting point of 213° melted at 212° . $[\alpha]_D^{25} = +8.8^\circ$.

Resolution of dl-Leucine with Papain—6.5 gm. of *dl*-leucine were

transformed into the carbobenzoxy compound according to the procedure described for the *l* compound (11). Carbobenzoxy-*dl*-leucine was a syrup, as was also the corresponding *l* compound. The carbobenzoxy-*dl*-leucine was dissolved in 44 cc. of *N* NaOH; then there were added 75 cc. of 2 *N* sodium acetate solution, 150 cc. of citrate buffer pH 5, 6.5 cc. of aniline, 1.5 cc. of glacial acetic acid, 0.5 gm. of cysteine, 150 cc. of papain solution, and water up to 500 cc. The mixture was incubated at 40° for 1 week, with occasional shaking. During this time, carbobenzoxy-*l*-leucine anilide had separated out in almost quantitative yield.

$$\begin{array}{ll} \text{C}_{20}\text{H}_{24}\text{O}_2\text{N}_2. & \text{Calculated. C 70.6, H 7.1, N 8.2} \\ 340.4 & \text{Found. " 70.4, " 7.4, " 8.4} \\ [\alpha]_D^{25} = & -23.5^\circ \text{ (4.5\% in glacial acetic acid)} \end{array}$$

The filtrate of the anilide was acidified with 5 *N* HCl and extracted with ether. The ethereal solution was extracted with potassium bicarbonate, the latter was acidified, the precipitated syrup was again extracted with ether, and the ether layer was evaporated *in vacuo*. Carbobenzoxy-*d*-leucine remained as a syrup. It was hydrogenated catalytically in methanol solution in the presence of HCl. After filtration and evaporation *in vacuo*, about 4 gm. of *d*-leucine hydrochloride remained in crystalline form; this represented an almost quantitative yield. It was transformed into the free *d*-leucine which, after one recrystallization, showed:

$$[\alpha]_D^{25} = \frac{-0.79 \times 2.3292}{1.1045 \times 0.1008} = -16.6^\circ \text{ (in HCl of 21\%)}$$

In the literature there have been recorded values varying from +15.5° to +16.9° for *l*-leucine and -16.9° for *d*-leucine (13-15).

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THE ESTIMATION OF CYSTINE IN FINGER NAIL CLIPPINGS WITH HYDROLYSIS FOR ONE HOUR*

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Sullivan and Hess (1) found that all the cystine in finger nail clippings was liberated by hydrolyzing the clippings with 20 per cent hydrochloric acid for 6 to 7 hours. This type of hydrolysis was used by Sullivan (2) and by Sullivan and Hess (3) for determining the cystine content of casein and various purified proteins and in general, with the exception of certain substances such as insulin in which the cystine is more or less labile, is considered very convenient and satisfactory. The hydrolysate can stand overnight with little if any change and then can be diluted, neutralized, and used for colorimetric or iodometric determination of cystine. Hydrochloric acid hydrolysates of finger nails require no decolorization.

From various quarters, in particular from hospital authorities interested in the possible relation of the cystine content of the finger nails to health and disease, came inquiries as to the possibility of shortening the time required for the determination of cystine.

With several purified proteins Sullivan and Hess (4) found that hydrolyzing for 2 hours in the presence of titanous chloride gave as good cystine values as did the 6 or 7 hour hydrolysis without titanium. The titanium treatment, though useful in shortening the time of hydrolysis, necessitated neutralizing and filtering to remove the titanium precipitate and washing this precipitate. In relatively careful hands the TiCl_3 treatment should prove very useful in giving good yields and in shortening the time necessary

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for hydrolysis. Finger nail clippings, however, were found more resistant than the purified proteins worked with. Thus in 2 hours hydrolysis in the presence of TiCl_3 a normal finger nail hydrolysate showed no cysteine or cystine by the Sullivan method, while by the Okuda (5) method 7.5 per cent of a cysteine complex was shown. In 4 hours hydrolysis in the presence of TiCl_3 the Sullivan method gave 12.6 per cent cystine, the Okuda 12.8. With 6 hours TiCl_3 hydrolysis both gave the same value 12.7.

Since with finger nail clippings the presence of TiCl_3 gave only a moderate shortening of the time necessary for full hydrolysis, other procedures were sought for. Finally, based on an observation of Salkowski (6) that horn substance could be hydrolyzed in 2 to 3 hours by sulfuric acid (2 volumes of concentrated acid, d 1.84, to 3 volumes of water) without loss of sulfur, a much shorter period of hydrolyzing finger nails was devised.

Short Hydrolysis—40 to 50 mg. of nail clippings finely cut and 0.15 cc. of 15 N H_2SO_4 made as described by Salkowski were heated in a small acetylation flask in an oil bath at 150° for 1 hour (Salkowski never allowed his digest to go beyond 126°). The dark hydrolysate of the finger nails was washed into a small beaker with 5 cc. of water and was decolorized by means of 25 mg. of acid-washed carbex E with gentle heating and filtering. The carbex E was washed with 3 cc. of 0.1 N hydrochloric acid and the total solution made to 25 cc. with water. The solution, now practically a 0.1 N acid, was used for cystine determinations. This method of hydrolyzing not only shortens the time but obviates the neutralization of the hydrolysate.

As reported by Sullivan and Howard,¹ the 1 hour sulfuric acid digest of finger nails of fourteen normals gave in undecolorized hydrolysates an average cystine content of 11.9 per cent, only slightly higher than the customary 7 hour hydrolysis employed by Sullivan and Hess, which gave an average value of 11.4 per cent cystine. So the concentrated H_2SO_4 hydrolysis without decolorization was considered satisfactory. Further work, however, with a larger number of cases, both normal and pathological, made this conclusion questionable. The shade of the color developed in the H_2SO_4 digest of the finger nails was at times

¹ Reported at the meeting of the Division of Medicinal Chemistry, American Chemical Society, Pittsburgh, 1936.

different from the standard, and often seemed to contain an extraneous color difficult to reduce by the sodium hyposulfite.

This extraneous color when present made the supposed cystine values considerably higher than given by the 7 hour hydrochloric acid hydrolysis and by the Okuda iodometric cystine method applied to the same hydrolysate. The foreign color came from the humin generated by the H_2SO_4 and was eradicated by the decolorization process. So treatment with a small amount of carbex E is a necessary part of the procedure.

TABLE I

Per Cent Cystine Content of Finger Nails of Pathological Cases

Series A, hydrolysis with 15.0 N H_2SO_4 for 1 hour; Series B, hydrolysis with 20 per cent HCl for 7 hours.

Case No.	Series A		Series B	
	Sullivan	Okuda	Sullivan	Okuda
1	9 3	9 4	9 2	9 8
2	9 4	9 5	9 5	9 1
3	9 0	9 1	9 2	9 2
4	10 2	10 8	10 1	10 3
5	9 6	9 8	9 9	10 2
6	9 2	9 8	9 4	9 6
7	10 1	10 7	10 8	10 9
8	12 1	12 4	12 2	12 4
9	9 3	9 0	8 7	9 8
10	10 0	10 6	10 2	10 1
11	10 9	11 3	11 6	11 9
Average	9 9	10 2	10 1	10 3

Since the demand for a shorter method came from hospitals and from physicians in general practise, the short method has been applied to the finger nail clippings from patients in hospitals or under a physician's care. However, when tested on a number of normals, it gave a value of 11.9 per cent cystine, in agreement with the longer HCl hydrolysis on sixteen cases which gave 11.5 per cent and in agreement with previous work of Sullivan and Hess (1) who obtained an average normal value of 11.69 per cent, with variation from 10.28 to 13.00 per cent.

As may be noted from Table I, the values obtained by the 1 hour H_2SO_4 hydrolysis agree well with those by the Okuda method

on the same hydrolysis and with those by the Sullivan and the Okuda on the longer HCl hydrolysis. In the cases listed the amount of nails used for each set of analyses (Series A and B) was small, 17 to 36 mg., a fact that makes any error in weighing the more noticeable than with larger samples. In Case 9 the value 8.7 in the long hydrolysis is obviously an error, but because of lack of material no repetition was possible.

If conserving time is a factor, the short H_2SO_4 hydrolysis may be useful, especially in finger nail work. In other fields, it is not as satisfactory as the longer HCl hydrolysis which in general work is still found preferable.

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FACTORS AFFECTING THE DETERMINATION OF INORGANIC IRON IN ANIMAL TISSUES*

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In previous papers (1-3) published from this laboratory, figures have been given for the available or ionizable iron in a number of biological materials. The ionizable iron as determined by the α, α' -bipyridine method in general showed good agreement with the values for available iron as determined by animal feeding. Because of these consistent results, we concluded that the bipyridine method was a useful means of estimating the nutritionally available iron in foods. However, when the values obtained by Shackleton and McCance (4) became available, which were considerably higher than those we had obtained, we felt it was advisable to study the bipyridine method in greater detail. Shorland and Wall (5) recently suggested that certain reducing agents tend to remove iron from hemoglobin, while reagents such as pyrophosphate do not attack hemoglobin iron.

Since some of the greatest discrepancies have been found in the case of liver and since this tissue is often used as a source of iron, we have studied the available iron content of liver by several modifications of the original method.

EXPERIMENTAL

All studies involving modifications of the method were made on livers removed from chicks or rats immediately before the determination was started. The animals were killed by decapitation, after which the entire liver was removed and washed with redis-

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tilled water. Two methods were used for the preparation of the tissue for analysis. In the first experiments the method previously employed in this laboratory was continued. It consists of weighing a definite amount of tissue (1 to 2 gm.), macerating the sample with chromium-plated scissors, and placing the macerated tissue in a 15 cc. graduated centrifuge tube containing 5 cc. of 2.5 per cent trichloroacetic acid. The reducing agent and bipyridine are then added and the suspension allowed to stand for 24 hours. After this period 5 cc. of 80 per cent alcohol are added, the tube centrifuged, and the clear liquid compared with a standard iron solution.

In later determinations, the livers were homogenized according to the method described by Potter and Elvehjem (6). A portion of the liver (3 to 5 gm.) was placed in a weighed tube containing about 5 cc. of 10 per cent trichloroacetic acid and weighed again. The contents were homogenized, diluted to definite volume, and an aliquot of the suspension taken for analysis. This method has several advantages; first, the tissue is reduced to very fine particles which allow complete extraction of the iron and, second, several aliquots containing the desired amount of available iron (15 to 20 micrograms) can be prepared at once. It might also be added that the use of the more concentrated trichloroacetic acid eliminates the difficulties due to incomplete precipitation of protein, which is often encountered when the lower concentration (2.5 per cent) of acid is used.

When macerated tissue was used, the total iron was determined in the usual way, but when the tissue was homogenized, aliquots of the suspension were transferred to digestion flasks and digested with sulfuric and perchloric acids. The total iron was determined according to the method of Lintzel (7).

The first modifications in the determination of available iron included the use of one of the following reducing agents: thioglycolic acid, hydroquinone, hydrosulfite, cysteine, or glucose. The following amounts were used: 3 drops of thioglycolic acid, 0.1 gm. of hydroquinone, a few grains of sodium hydrosulfite and cysteine, and 2 cc. of a 25 per cent solution of glucose. When homogenized tissue was used, the reducing agent was added directly to the suspension, which was then heated on a water bath at 85–90° for 5 minutes. The contents of the tube were

cooled, centrifuged, and the clear liquid poured off. The solution containing the extracted iron was brought to between pH 3 and 5 with iron-free NH_4OH . 1 cc. of 0.2 per cent α, α' -bipyridine was added, and the solution diluted to a definite volume and compared with a standard iron solution.

TABLE I
Effect of Various Reducing Agents on Available Iron in Liver

Liver from chick No.	Treatment	Reducing agent	Available Fe per gm. fresh tissue mg.
2667	Macerated	Hydroquinone	0.014
	"	Thioglycolic acid	0.016
2628	"	Hydroquinone	0.049
	"	Thioglycolic acid	0.043
26	"	Hydroquinone	0.053
	"	Glucose	0.042
30	Homogenized	None	0.079
	"	Glucose	0.08
	"	Hydroquinone	0.079
	"	1 cc. thioglycolic acid	0.077
31	"	None	0.051
	"	Hydroquinone	0.050
	"	1 cc. thioglycolic acid	0.052
35	"	None	0.066
	"	3 drops thioglycolic acid	0.066
	"	7 " " "	0.066
	"	10 " " "	0.066
37	"	5 " " "	0.041
	"	Cysteine	0.040
39	"	None	0.023
	"	Hydrosulfite	0.022
	"	5 drops thioglycolic acid	0.023

Typical results obtained with different reducing agents are shown in Table I. A few results with thioglycolic acid and hydroquinone on macerated tissue are included to show the variable results. These variations were due mainly to the interference of the yellowish flavin extracted from the liver. Sodium hydrosulfite reduced the flavin to the leuco form as well as reducing the iron, but under the conditions employed hydroquinone or thioglycolic acid did not reduce the flavin, which has a very negative

oxidation-reduction potential, and therefore did not prevent the yellow color from interfering in the final comparison of the colored iron bipyridine. In the case of hydroquinone a brownish color often developed if the readings were not made immediately.

The results with homogenized tissue were much more constant. Part of this may be due to the more uniform preparation of each sample. The amount of ionizable iron in all samples from the same liver checked very well, regardless of the reducing agent used. In fact, fair results were obtained when no reducing agent was added. Undoubtedly the reducing systems present in the homogenized tissue were able to bring about a reduction of the iron. However, the yellow color due to flavin was evident in the samples in which no reducing agent was added and where glucose, hydroquinone, or cysteine was used. Thioglycolic acid reduced flavin in the presence of tissue when the suspension was heated. Thioglycolic acid in the absence of tissue will not reduce flavin. The best reducing agents for the elimination of the flavin interference are sodium hydrosulfite and thioglycolic acid. The extraction of iron is complete because repeated extractions gave no indication of additional iron.

Since Shorland and Wall (5) have shown that the non-hemoglobin Fe content of blood is increased when thioglycolic acid is used as the reducing agent, we were interested in studying the effect of this reagent and the other reducing agents used in this work on hematin added to the homogenized liver sample and on whole blood. The hematin used was a highly purified preparation which was practically free from inorganic iron. The amount of hematin added to each sample was equivalent to 0.095 mg. of Fe. The results of this work are summarized in Table II. It will be noted that hydrosulfite and cysteine had no effect on the Fe of hematin, whereas some of the iron was liberated from the hematin molecule when thioglycolic acid was used. More iron was liberated as the amount of reagent used was increased. Similar results were obtained when thioglycolic acid was used on whole blood. Apparently this reducing agent has the ability of removing some of the iron present in both hematin and hemoglobin. This result confirms those obtained by Shorland and Wall with thioglycolic acid. However, from Table I it will be seen that thioglycolic acid has no such effect on the organic iron found in liver under

these conditions. It is also evident from the results with blood that a reducing agent such as hydrosulfite is necessary in order to recover the inorganic iron quantitatively.

In the determination of the inorganic iron of blood, Shorland and Wall used $\text{Na}_4\text{P}_2\text{O}_7$, as suggested earlier by Tompsett (8), to liberate what Barkan (9) terms the "easily separable" iron of blood. They found, using this reagent, that the results for in-

TABLE II

Effect of Reducing Agents on Hematin Added to Liver Samples and on Blood
0.095 mg. of Fe in hematin added to liver samples.

Chick No.	Reducing agent	Available Fe in liver sample	Available Fe found	Fe removed from added hematin
		mg.	mg.	mg.
34	None	0.08	0.080	None
	10 drops thioglycolic acid	0.08	0.108	0.028
36	3 " " "	0.124	0.144	0.02
	7 " " "	0.124	0.151	0.027
	10 " " "	0.124	0.158	0.034
37	5 " " "	0.064	0.086	0.022
	Cysteine	0.064	0.063	None
38	$\text{Na}_2\text{S}_2\text{O}_3$	0.025	0.026	"
	5 drops thioglycolic acid	0.025	0.052	0.027
		Inorganic Fe per 100 cc. blood		
		mg.		
60	None	1.01		
	$\text{Na}_2\text{S}_2\text{O}_3$	1.57		
	2 drops thioglycolic acid	2.57		
61	None	1.65		
	$\text{Na}_2\text{S}_2\text{O}_3$	1.93		
	2 drops thioglycolic acid	3.30		

organic iron of blood were considerably lower than those obtained by other methods. They found further that when blood was allowed to age in contact with $\text{Na}_4\text{P}_2\text{O}_7$, the inorganic Fe was further decreased.

We have also studied the effect of using $\text{Na}_4\text{P}_2\text{O}_7$ in addition to the reducing agents for both liver and blood. Typical results are presented in Table III. It will be noted that low results were

obtained in all samples of liver except those in which thioglycolic acid was used as the reducing agent. When the amount of

TABLE III
Effect of $\text{Na}_4\text{P}_2\text{O}_7$ on Available Iron of Liver and Blood

Samples	Chick No.	Reducing agent	Available Fe per gm. fresh tissue		
			No $\text{Na}_4\text{P}_2\text{O}_7$	10 per cent $\text{Na}_4\text{P}_2\text{O}_7$	
			mg.	cc. $\text{Na}_4\text{P}_2\text{O}_7$	mg.
Liver, macerated	1001	Hydroquinone	0.062	1	0.033
	1002	"	0.032	1	0.015
	2628	Thioglycolic acid	0.043	1	0.043
	1005	" "	0.058	1	0.059
Liver, homogenized	33	" "	0.039	1	0.039
	57	" "	0.043	2	0.044
		Hydrosulfite	0.043	1	0.031
		"	0.043	2	0.019
			mg. per 100 cc.		mg. per 100 cc.
Chicken blood	60	None	1.01	1	0.17
		Hydrosulfite	1.57	1	1.38
		Thioglycolic acid	2.57	1	2.60
	61	None	1.65	1	0.57
		Hydrosulfite	1.93	1	1.87
		Thioglycolic acid	3.30	1	3.52

TABLE IV
Effect of $\text{Na}_4\text{P}_2\text{O}_7$ on Standard Fe Solution in Various Concentrations of Acid

Trichloroacetic acid	Treatment of sample	10 per cent $\text{Na}_4\text{P}_2\text{O}_7$	Fe present	Fe recovered
per cent		cc.	mg.	mg.
2.5	None	1	0.050	0.050
2.5	"	2	0.050	0.050
2.5	Heat and centrifugation	1	0.05	0.039
2.5	" " "	2	0.05	None
5	None	1	0.05	0.05
5	Heat and centrifugation	1	0.05	0.014
10	None	1	0.05	0.05
10	Heat and centrifugation	1	0.05	0.011
20	None	1	0.05	0.05
20	Heat and centrifugation	1	0.05	None

$\text{Na}_4\text{P}_2\text{O}_7$ added was increased, still lower results were obtained. In the case of blood the use of $\text{Na}_4\text{P}_2\text{O}_7$ gave the lowest results

when the blood was extracted and centrifuged before the addition of a reducing agent. The $\text{Na}_4\text{P}_2\text{O}_7$ had only a small inhibitory effect when $\text{Na}_2\text{S}_2\text{O}_3$ was added before extraction, and with thio-glycolic acid as the reducing agent the inhibitory effect was not observed. In an attempt to explain these results, the effect of $\text{Na}_4\text{P}_2\text{O}_7$ on a solution of inorganic iron was studied. Solutions of inorganic iron in various concentrations of acid were prepared and the effect of $\text{Na}_4\text{P}_2\text{O}_7$ on the recovery of iron from these solutions determined. Results of these trials are presented in

TABLE V
Available Fe in Liver

Whole liver, homogenized in 10 per cent CCl_3COOH ; sample heated on a water bath 5 minutes; reducing agent, $\text{Na}_2\text{S}_2\text{O}_4$.

Chick No.	Available Fe per gm. dry liver	Total Fe per gm. dry liver	Availability
	mg.	mg.	per cent
40	0.104	0.212	49
41	0.182	0.345	53
42	0.098	0.202	48
43	0.107	0.193	55
44	0.096	0.164	58
45	0.061	0.125	58
Rat No.			
6131	0.102	0.18	56
6133	0.21	0.32	66
6134	0.151	0.242	63
6135	0.079	0.156	51
6121	0.143	0.265	54
6122	0.26	0.52	50

Table IV. It will be noted that when the solutions were heated and centrifuged before the bipyridine reagent was added, low results were obtained. Lower results were obtained with increasing concentration of acid or with larger amounts of $\text{Na}_4\text{P}_2\text{O}_7$. However, when the solutions were not centrifuged and the bipyridine and reducing agent were added directly to the iron and pyrophosphate solutions, 100 per cent recovery was obtained.

From these results it is evident that the low results obtained when $\text{Na}_4\text{P}_2\text{O}_7$ is used in determining the inorganic iron of blood

or liver are due to the formation of acid-insoluble iron pyrophosphate which remains in the residue upon centrifuging or filtering. The fact that $\text{Na}_4\text{P}_2\text{O}_7$ has no effect when thioglycolic acid is used may be due to the iron combining first with the thioglycolic acid and thus preventing formation of the insoluble iron pyrophosphate.

In Table V are listed some of the typical results of iron analysis of chick and rat livers by the method which involves the use of the

TABLE VI
Recovery of Fe Added to Liver Sample

Fe added, 0.020 mg.

Available Fe of sample	Available Fe found	Fe recovered	Recovery
mg.	mg.	mg.	per cent
0.174	0.195	0.021	105
0.045	0.064	0.019	95
0.031	0.051	0.020	100
0.032	0.051	0.019	97

TABLE VII
Effect of Storage on Available Iron of Liver

Liver		Temperature, 4°				Temperature, 20°			
		Available Fe per gm. dry liver	Percent available	Change in availability	Total Fe	Available Fe	Percent available	Change in availability	Total Fe
		mg.		per cent	mg.	mg.		per cent	mg.
Pig	Start	0.265	74		0.36	0.265	74		0.36
	8 days	0.252	70	-4	0.366	0.280	72	-2	0.39
	16 "	0.255	64	-10	0.40	0.240	68	-6	0.353
Calf	Start	0.332	69		0.475	0.332	69		0.475
	8 days	0.308	68	-1.5	0.455	0.316	66	-3	0.48
	16 "	0.275	65	-4.5	0.42	0.323	64	-5	0.5

homogenized tissue and heating for a short period of time, as previously described. A large number of livers of both rats and chicks have been analyzed for inorganic iron in connection with other work, giving results which fall well within the range of those listed in Table V. The method has also been applied to the determination of inorganic iron in other tissues such as skeletal muscle and kidney with equally satisfactory results. Recovery

experiments with iron added as ferrous ammonium sulfate give an indication of the efficiency of the method. Typical results are given in Table VI.

These results, although not in agreement with those reported by Shackleton and McCance, further substantiate the earlier work from this laboratory showing that less than 70 per cent of the iron in liver is present in the inorganic form. That all the iron is not in the inorganic form follows from the fact that a part of it is contained in such hematin compounds as hemoglobin, cytochrome, and catalase. The high results of Shackleton and McCance may be due to the liberation of some of the iron of these hematin compounds by autolysis on standing. However, we have found that both pork and beef liver, when allowed to stand at a temperature of 4° or 20°, show no increase in inorganic iron content. In fact, there is a slight decrease in availability. These results are listed in Table VII.

In conclusion it may be said that this work does not change the previous results on the availability of iron in various animal tissues that have been obtained in this laboratory. However, the use of heat and homogenization does tend to simplify the determination of inorganic iron in liver and other tissues and permits greater accuracy.

SUMMARY

1. A modified method for the estimation of inorganic iron in animal tissues involving the use of homogenization and heat is described.

2. The effect of various reducing agents has been studied.

3. The low results for inorganic iron in liver and blood when $\text{Na}_4\text{P}_2\text{O}_7$ is added are due to partial precipitation of the iron as iron pyrophosphate.

4. Further evidence that only a part of the iron in liver is in the inorganic form is presented.

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OBSERVATIONS ON THE PROTEOLYTIC ACTIVITY OF THE SERA OF DOGS WITH EXPERIMENTAL UREMIA

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Becher (1) has suggested that the increased nitrogen metabolism often observed in renal insufficiency (2, 3) may be brought about, in part, by retention of proteolytic ferments. Bradford (4) showed that dogs deprived of three-fourths of their renal tissue displayed elevated nitrogen excretion, nitrogen retention, and loss of body weight attributed to accelerated protein catabolism. Similar increases in catabolism of body protein occur when nitrogen retention is the result of extrarenal causes, *e.g.* extreme dehydration (5). It is the purpose of this paper to report the results of observations on the proteolytic activity of the sera of normal dogs and of dogs with experimental uremia produced by bilateral ureteral ligation.

Methods

Proteolytic activity of sera was studied by a method similar to that employed by Fuchs (6) in the serological diagnosis of malignancy, in which the increase in non-protein nitrogen upon incubating serum with a fibrin substrate is taken as a measure of proteolytic activity. The modified procedure we have employed is as follows:

A stock preparation of fibrin was obtained by defibrinating normal dog blood. The fibrin was washed free of gross blood, wrapped in a pledget of cotton, suspended in cold running water for 12 hours, dried in a vacuum desiccator, ground in a mortar to a fine powder, and stored in the ice box in an evacuated container. Fibrin preparations other than the stock powder were similarly prepared.

Blood samples were drawn with aseptic technique, defibrinated, or allowed to clot in sterile tubes. When the stock fibrin preparation was employed, the serum was immediately used. In instances in which the same dog's fibrin was used as substrate, the sterile serum was kept in the ice box until the fibrin could be prepared. 5 mg. of fibrin powder were placed in a 15 cc. graduated centrifuge tube, 1 cc. of serum added and mixed, and the stoppered tube incubated 8 hours at 38. Similar tubes containing serum only and fibrin plus 1 cc. of water were also prepared and incubated. All three tubes were then made to 12 cc. volume with 5 per cent trichloroacetic acid, and after standing about 30 minutes the contents were filtered. The contents of a second series of three tubes prepared exactly as the first series were precipitated without incubation in order to serve as blanks. Total nitrogen was determined in aliquots of the protein-free filtrates by the manometric micro-Kjeldahl procedure, with the improved reagents of Van Slyke and Kugel (7). All incubations, Kjeldahl determinations, etc., were carried out in duplicate. These analyses permitted the following calculations: non-protein nitrogen in serum + fibrin (digested) minus non-protein nitrogen in serum + fibrin (blank) = total proteolysis.

The total proteolysis is the sum of (1) autolysis of serum only, (2) autolysis of fibrin only, and (3) proteolysis of serum-fibrin contact. Hence, total proteolysis minus (serum autolysis + fibrin autolysis) = serum-fibrin proteolysis. The results are expressed as mg. per cent increase in non-protein nitrogen in the mixture incubated.

Similar observations were made on uremic dogs 3 or 4 days following bilateral ureteral ligation. In most instances a stock fibrin was employed as a substrate; however, in other instances the dog's own fibrin was used. In several experiments incubation of separate samples of serum and fibrin was omitted, so that the corrections for serum and fibrin autolysis could not be made, and only the calculated "total proteolysis" is reported.

Results

The results of seven experiments with sera of five normal dogs and of fourteen experiments with sera of nine uremic dogs are summarized in Table I. The total proteolysis occurring upon

incubation of normal dog serum with fibrin is only slightly greater than the experimental error of the method. In these instances the total proteolysis seems to be the sum of approximately equal quantities of nitrogen arising from serum and fibrin autolysis and the proteolysis of serum-fibrin contact. In two instances when the

TABLE I

Proteolytic Activity of Normal and Uremic Dog Sera (Dog Fibrin Substrate)

Dog No.	Serum non-protein N	Total proteolysis	Fibrin autolysis	Serum autolysis	Serum fibrin proteolysis	Fibrin substrate	Remarks
	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent		
1	31	0.64	0.56	0.44	-0.36	Stock	Normal dog
1	30	0.52	0.00	0.16	0.36	Own fibrin	" "
2	33	2.00	0.35	1.80	-0.15	Stock	" "
3	20	1.06	0.31	0.58	0.17	"	" "
3	20	1.04	0.12	0.58	0.34	Own	" "
4	30	2.46	0.27	1.68	0.51	Stock	" "
5	29	1.46	0.38	0.56	0.62	"	" "
Days after ureteral ligation							
1	224	10.98	1.32	1.26	8.40	Stock	4
1	224	7.35	0.57	1.50	5.28	Own	4
2	281	23.70	0.72	2.92	20.06	Stock	4
3	199	9.48	0.56	1.38	7.54	"	4
3	199	9.82	1.87	2.40	5.55	Own	4
4	270	13.95	0.50	-1.80	15.25	Stock	4
4	270	3.00	0.10	0.90	2.00	Own	4
6	171	17.52				Stock	3
6	171	16.52				Own	3
7	143	11.80				Stock	3
7	143	13.80				Own	3
8	132	21.10				Stock	3
8	132	14.40				Own	3
9	166	15.00				Stock	3

dog's own fibrin was used as substrate, the character of the proteolysis was not altered.

The proteolytic activity of uremic serum was found to be markedly increased in all instances save one. Although only total proteolysis was measured in half of the experiments, the results when fibrin and serum autolysis were simultaneously measured

indicate that the increased proteolytic activity is largely the consequence of serum-fibrin contact. Use of the dog's own fibrin as substrate in these instances did not affect the intensity of the proteolytic process.

These results point to an increase in the quantity of proteolytic ferment (or ferments) in the circulation in experimental renal insufficiency. Whether these ferments arise from the pancreas and intestinal wall, from leucocytes, or whether they are hydrolases of the peripheral tissue cells which have escaped into the circulation to be retained there as a result of renal insufficiency is not known.

It is strange that the autolytic activity of the serum of uremic dogs is not greatly increased, and that any substrate other than the serum itself is required to demonstrate the increased proteolytic action. This would suggest that the proteolysis is due to ferments adsorbed on and added with the fibrin, as has been shown by Schmitz (8); however, this cannot be the case, as the uremic sera yield high values for proteolysis with the same stock fibrin used in control measurements, and because heating the serum abolished the augmented proteolytic action against unheated fibrin.

Whether the accumulation of proteolytic ferments measurably augments the increase in non-protein nitrogen in body fluids in states of renal insufficiency cannot be stated. The absence of marked serum autolysis in these experiments makes it seem unlikely that it is a significant factor.

It is evident that the Fuch's serological test for malignancy is positive when applied to sera from uremic dogs with normal dog fibrin. We have not applied the test to sera from patients with uremia.

SUMMARY

Serum from dogs with experimental uremia incubated with normal dog fibrin exhibits a greater proteolytic action than does normal dog serum under the same conditions.

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DETECTION AND QUANTITATIVE DETERMINATION OF SMALL AMOUNTS OF GLUCOSE IN MIXTURES CONTAINING MALTOSE

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In the analysis of hydrolytic cleavage products of glycogen or starch, such as occur in tissue extracts and in diastatic reaction products in general, we were confronted with the task of differentiating between glucose and maltose, both qualitatively and quantitatively. Our studies called for a method that permits the detection and quantitative determination of slight amounts, often but fractions of a mg., of glucose in the presence of relatively much maltose, as well as the determination of small quantities of maltose in media which contain variable quantities of glucose.

A basis for such a method was found in the great difference between the rate of fermentation of the two sugars at alkaline reaction. In a previous paper (1) we described a simple titrimetric technique which is suitable for the observation of the fermentation rate of glucose at moderately alkaline reactions. When attempting to apply the same technique to maltose, we found that, in contrast to glucose, the fermentation of this sugar is wholly suppressed when the pH of the medium is raised to from 7.5 to 8.0.

Qualitative Test for Glucose

Prepare a 20 per cent alkaline yeast suspension by rubbing up 10 gm. of commercial bakers' yeast in water and making up the volume to approximately 50 cc. Add 1 cc. of phenol red indicator (0.06 per cent aqueous solution) and 0.1 M Na_2CO_3 , drop by drop with continuous stirring, until the pink color persists for about 1 minute.

Measure into a test-tube 5 cc. of the unknown (presumably mixed) sugar solution to be tested for glucose. Add 1 drop of

phenol red indicator and enough 0.01 M Na_2CO_3 to adjust the reaction to slight alkalinity (pink color, pH 7.2 to 7.4). In another test-tube of approximately the same diameter, 5 cc. of a 0.1 per cent maltose solution are made alkaline in the same manner; this serves as control.

Introduce simultaneously 5 cc. portions (use a graduated cylinder for measuring) of the slightly alkaline yeast suspension into the two test-tubes containing the unknown sugar solution and maltose, respectively. Close with rubber stoppers, mix by inversion, and allow to stand for about 5 minutes. At the end of this time run from a burette into each of the tubes 0.01 M Na_2CO_3 until the original pink color is restored. The operations in the two test-tubes should be performed as simultaneously as possible; for this reason the author prefers to use two burettes and the help of an assistant in this work. If the unknown sugar solution contains glucose, it requires in the titration distinctly more carbonate solution than the control which contains only maltose. The difference between the two titrations not only shows the presence of glucose, but, as we have shown previously (1), furnishes a fair measure of its quantity.

The presence of glucose in the solution under examination is revealed before titration, in that the pink color of the indicator fades and turns brown and yellow in it much faster than in the control tube, which contains no glucose.

The yeast suspension and carbonate solution described are suitable only when the amount of glucose in 5 cc. of solution is at least 2 mg. The smaller the quantity of glucose to be detected, the more dilute the yeast suspension and the carbonate solution must be. Thus, when the reagents are used in 10-fold dilution, *i.e.*, a 2 per cent yeast suspension for fermentation and 0.001 M carbonate for titration, 0.5 mg. of glucose can still be detected with security, irrespective of the amount of maltose and of non-fermentable reducing matter present.

We have employed this titrimetric technique for following the rate of fermentation of various sugars at pH 7.2 to 8.0. In this procedure the control tube contains water in place of the sugar solution. As soon as the color of the indicator fades perceptibly, carbonate is added from the burette to restore the initial pink color. The control tube is treated in the same manner by an

assistant, in order to obtain correction for the self-fermentation of yeast. By reading the burettes at regular intervals, at each minute for example, figures are obtained that represent the rate of fermentation as reliably as data obtained with the aid of more elaborate apparatus. The rate of fermentation is a useful characteristic for the identification of sugars.

Quantitative Determination

Since pH control in the manner described while fermentation is in progress is not practicable, we examined the effect of varying

TABLE I

Effect of Varying Concentrations of Na_2CO_3 on Rate of Fermentation of Glucose and Maltose at 25°

The sugars are given as mg. per 100 cc. of solution; maltose is expressed as the glucose equivalent of the copper reduced by it.

Fermentation period	Na_2CO_3 added per 100 cc fermentation mixture			
	0 gm	0.03 gm	0.05 gm.	0.08 gm.
Glucose recovered as unfermented residue				
min.				
0	40 4	40 4	40 4	40 4
3	13 2	29 6	33 0	33 2
6	0	10 8	23 9	25 9
10	0	1 6	5 6	17 7
Maltose recovered as unfermented residue				
0	42 0	42 0	42 0	40 1
5	39 9	41 5	41 9	40 2
10	37 7	41 4	41 7	40 1
20	33 6	41 4	42 0	40 2
60	24 3	41 0	41 8	40 3

amounts of Na_2CO_3 , added in advance, upon the rate of fermentation of glucose and maltose. Of each of the two sugars four batches, each 200 cc., were set up with 20 gm. of washed yeast; one of each group was fermented without the addition of Na_2CO_3 , the others with varying amounts of carbonate. Periodically samples were withdrawn for the determination of unfermented sugar. The results, given in Table I, show that the rate of fermentation of glucose progressively diminishes as the amount of added Na_2CO_3 is augmented. The fermentation of maltose, slow enough

in the absence of carbonate, is virtually stopped by as little as 0.03 per cent of Na_2CO_3 . This, of course, is not a pattern of general validity; there are, namely, batches of yeast which have a considerable carbohydrate reserve and consequently such a vigorous self-fermentation that a small amount of carbonate is neutralized in a short time by the CO_2 derived from self-fermentation plus glucose fermentation, whereupon the fermentation of maltose begins. Fortunately, the rate of fermentation of glucose is still fast enough when Na_2CO_3 is added in an excess sufficient to safeguard the maintenance of an adequate degree of alkalinity.

TABLE II

Selective Fermentation of Glucose in Presence of Maltose, in Solutions Containing 0.1 Gm. of Na_2CO_3 and 10 Gm. of Washed Yeast per 100 Cc., at 25°

The sugars are given as mg. per 100 cc. of solutions; maltose is expressed as the glucose equivalent of the copper reduced by it.

Fermentation period	Glucose in mixture				
	40.4	0	30.3	20.2	10.1
	Maltose in mixture				
	0	40.8	10.2	20.4	30.6
Recovered as unfermented residue					
min.					
0	40 4	40 8	40 5	40 6	40 7
5	25 8	40 2	29 1	32 2	35 6
10	14 1	40 1	20 6	26 7	33 0
15	7 3	40 2	15 5	23 1	31 7
20	2 1	40 3	12 3	21 7	31 0
30	0	40.3	10.7	20.3	30.2
90	0	40 5	9 9	19 9	29 9

In the experiment recorded in Table II we added 0.1 gm. of Na_2CO_3 to 100 cc. of sugar solution and yet, as may be seen in the first column, 40 mg. per cent of glucose were completely fermented within 30 minutes. In mixtures containing glucose and maltose in changing proportions, the glucose was completely removed and the maltose quantitatively recovered after 30 minutes of fermentation. Thus, one can allow in the selective fermentation of glucose and maltose ample safety margins both as regards the amount of added Na_2CO_3 and the time necessary for the complete fermentation of glucose.

Analytical Procedure

Into a 150 × 16 mm. Pyrex test-tube measure approximately 15 cc. of a 15 per cent suspension of washed yeast (10 gm. of yeast distributed in 100 cc. of water), centrifuge, decant, drain the supernatant water, and soak up the moisture adhering to the wall of the tube with a strip of filter paper. Introduce 15 cc. of the sugar solution, immediately followed by 1 cc. of a 1.6 per cent Na_2CO_3 solution; then stir up the yeast with a glass rod. The solution must not contain more reducing matter than corresponds, with respect to copper-reducing power, to 40 mg. per cent of glucose. Allow to ferment for 30 minutes, preventing the sedimentation of the yeast by occasional inversions of the stoppered tube. If the room temperature is below 25°, place the tube in a beaker of water with a temperature of 25–30°. Centrifuge, decant most of the clear supernatant fluid, and use 5 cc. portions for sugar determination. The difference between the reduction of the unfermented solution and the reduction after fermentation corresponds to the glucose in the mixture. The residual reduction represents maltose, provided, of course, that glucose and maltose were the sole reducing substances present. Otherwise, an additional fermentation is necessary to remove completely both the glucose and the maltose. To this end the original solution is fermented in the manner described, but without the addition of alkali, over a period of 2 to 2.5 hours. The residual reduction after this operation originates from reducing substances other than sugar, or from non-fermentable polysaccharides, or both. If A represents the total reduction of the solution, B the reduction after fermentation in alkaline medium, and C the reduction after the unmodified fermentation for 2.5 hours (non-fermentable reducing substances), then $A - B = \text{glucose}$ and $B - C = \text{maltose}$.

Addition of phenol red to the sugar solution as an indicator is a useful safeguard in the process of selective fermentation. Should one encounter a batch of yeast with such an extreme degree of self-fermentation as would tend to break down within 30 minutes the safety margin of alkalinity provided in the procedure, the fading of the red color of the indicator would serve as a warning. In such instances, in order to maintain the alkalinity of the solution, Na_2CO_3 must be added, a few mg. at a time, as the need for it arises.

Copper Reagent for Determination of Slowly Oxidized Sugars

In the final step of the analysis, *i.e.* in the determination of the copper-reducing power of mixtures of several sugars, care must be taken that each of the sugars be as completely oxidized in the presence of the others as if it were the sole reducing substance in the solution.

It is known that the rate of oxidation of various sugars shows great differences with any given reagent, and also that increase in alkalinity accelerates the reaction (2). Thus, the oxidation of maltose is slower than that of glucose and, as will be reported later, non-fermentable reducing polysaccharides that are always present among the enzymatic cleavage products of starch and of glycogen are oxidized at even a far lower rate than maltose. In order to accomplish the complete oxidation of the slowest reacting sugar within a reasonable length of time, it was found necessary to add to the Shaffer-Somogyi series of solutions a highly alkaline copper reagent, which has the following composition:

Na_2CO_3	25 gm.
Rochelle salt	25 "
NaOH , 1.0 N	40 cc.
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	6 gm.
KI	5 "
Na_2SO_4 *	200 "
KIO_3 , 1.0 N	15 cc.

* The inclusion of Na_2SO_4 is of recent origin. The smallest amount of glucose that can be determined with this "high alkalinity" reagent in the absence of Na_2SO_4 is 0.1 mg. By incorporation of the salt, its useful range is extended to 0.02 mg. of glucose.

The reagent is prepared as previously described (3). It is a stable solution, yet it must not be unduly exposed to air, in order to avoid the absorption of CO_2 and the consequent decrease of alkalinity.

The analytical procedure to be followed with this reagent does not differ from that given for the other Shaffer-Somogyi solutions. It requires for the complete oxidation of glucose a heating period of 10 minutes, for maltose 15, for the non-fermentable polysaccharides mentioned above 20 minutes. Thus, if the last named

reducing substances are present in a mixture of sugars, heating for less than 20 minutes leads to erroneous results.

In Table III are given, in terms of 0.005 N thiosulfate, the reduction equivalents of known amounts of glucose after heating periods of 10 to 20 minutes; with the aid of these figures a curve can be constructed from which the glucose equivalents corresponding to any reduction (titration) value can be read off or conveniently tabulated.

TABLE III

Titration Figures (Reduction Values) Corresponding to Known Amounts of Glucose Obtained with a High Alkalinity Copper Reagent

Heating period, 20 minutes.

Glucose in 5 cc. solution	Titration figures, 0.005 N thiosulfate	Glucose in 5 cc. solution	Titration figures, 0.005 N thiosulfate
mg.	cc.	mg.	cc.
0 03	0 15	0 25	1 52
0 05	0 27	0 30	1 82
0 10	0 58	0 50	3 12
0 15	0 88	1 00	6 50
0 20	1 15	2 00	13 17

SUMMARY

A test for the detection of small quantities of glucose in the presence of maltose and of non-fermentable reducing substances is described.

A method is given for the quantitative determination of small quantities of glucose and maltose in mixtures which contain both sugars.

A copper reagent is described which, due to its high degree of alkalinity, is suited for the analysis of sugars which have low rates of oxidation.

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THE RELATION OF PROTEIN TO HEMOGLOBIN BUILDING*

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The evaluation of dietary proteins for hemoglobin formation in nutritional anemia, when amounts of copper and iron adequate for optimal regeneration are fed, has received little consideration. Indeed, the extensive use of liver preparations in the treatment of secondary anemias makes a systematic study of this problem desirable.

The hematopoietic action of tryptophane has been reviewed by Kotake (1). Fontes and Thivolle (2) claim to have increased the hemoglobin content of the blood of healthy dogs and rabbits by the injection of tryptophane and histidine. Rats that were fed a tryptophane-deficient diet were found to be anemic after a period of 10 weeks. When dogs were made anemic by repeated bleedings, a response was obtained by injecting copper and iron (3). However, Fontes and Thivolle obtained the most rapid hemoglobin regeneration when tryptophane, histidine, copper, and iron were administered simultaneously. In contradistinction to these results, Tochowicz (4) concluded that tryptophane and histidine did not play an important rôle in hemoglobin formation in either hemorrhagic or pernicious anemia. His assumptions were based on a careful study of the variations of tryptophane and histidine in the blood of patients suffering from hemorrhagic or pernicious anemia, as compared with the values found for healthy individuals.

Feeding experiments on rats and mice (5) with tryptophane-deficient diets showed that after 25 to 30 days on such a regimen

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there was a decline in hemoglobin. With the addition of 0.2 per cent of tryptophane to such a diet the hemoglobin values returned to normal within a week or 10 days. Alcock (6) attempted to repeat the work of Fontes and Thivolle (2), making animals anemic by feeding a tryptophane-deficient diet. He used diets in which the protein was furnished by equal parts of zein and acid-hydrolyzed casein, or by the latter only. During a period of 5 months there was a loss in body weight of from 25 to 50 per cent, but there was no significant change in the hemoglobin values. In a second series, rats were made severely anemic by being fed an exclusive milk diet from the time of birth. The animals were then divided into two groups; the first group was fed a tryptophane-deficient diet without supplement, and the second group received the same diet plus 0.5 per cent of tryptophane. The rate of increase in hemoglobin was virtually the same in both groups, with the values approximating the normal at the end of about 3 weeks. The animals receiving tryptophane gained in weight, while the deficient group were emaciated and moribund after 3 weeks.

Extensive studies of the influence of diet and other factors on hemoglobin regeneration in hemorrhagic anemia have been made by Whipple and associates, the results being summarized in his Nobel prize lecture (7). Their work was carried out with dogs which were made anemic by repeated bleedings. The food factor to be assayed was then added to the basal diet fed these anemic animals. The increased hemoglobin formed on the supplemented diet above that on the basal diet was then calculated. They obtained a more rapid response with liver than with any other food substance. It should be pointed out, however, that the response in many cases was practically as good when iron alone was added to the basal diet as when liver was fed. The results of the work of Whipple and associates in the treatment of hemorrhagic anemia with liver have without doubt been an important factor in promoting the use of liver preparations for the treatment of various secondary anemias. Aside from hemorrhagic anemia, the extensive application of liver to the treatment of anemias should be looked upon with doubt, as the favorable response from its use may be due to its effect on the regeneration of constituents of the blood other than hemoglobin. The importance

of liver preparations is, of course, fully recognized in the treatment of pernicious anemia. Furthermore, in view of the excellent response obtained by adding iron alone, it seems highly probable that the hemorrhagic method of evaluating various foods in respect to hemoglobin formation is measuring, at least in part, the available iron. Certainly, if accurate information is to be obtained on the hematopoietic properties of foods, aside from the iron and copper they contribute, then adequate amounts of these elements should be fed to permit optimal hemoglobin formation.

The investigations reported in this paper were designed to ascertain whether the quality and level of proteins are factors in the rate of hemoglobin regeneration in nutritional anemia when the intake of iron and copper is adequate to permit optimal response.

EXPERIMENTAL

All of the animals used in these studies were young rats made severely anemic on an exclusive milk diet according to the technique described by Elvehjem and Kemmerer (8). At 5 to 7 weeks of age the hemoglobin values usually declined to between 3 and 4 gm. per 100 ml. of blood. In a few instances some of the animals had hemoglobin values slightly lower or higher. Blood was obtained from the tail and the hemoglobin was determined by the Newcomer method with a standardized disk. In order to follow closely the regeneration curve the hemoglobin was determined at 4 day intervals.

Diets—The diets employed are presented in Table I. During both the depletion period on the milk diet and while on the experimental diets the animals were fed *ad libitum*. Proteins from nine different sources were fed at a level intended to furnish approximately 17.5 per cent protein. Casein and liver were also fed at a level that furnished slightly less than 5 per cent of protein. The small amount of protein furnished by the vitamin addenda is not a complicating factor, as all groups of animals received the same supplement. Blood was obtained by exsanguination of healthy rats and evaporated on dextrin at a temperature of 50° in the proportion of 112 ml. of blood to 100 gm. of dextrin. Reprecipitated casein was used. Gliadin was prepared by extracting wheat gluten with 70 per cent ethyl alcohol. The

TABLE I
Percentage Composition of Diets

Diet No.	100-P	101-P	110-P	111-P	112-P	117-P	118-P	119-P	120-P	121-P	122-P	123-P
Dextrin	76 0	89 0	86 6	67 0	94 0	76 0	76 0	52 0	76 0	53 0	70 3	76 0
Blood.	18 0	5 0					18 0			41 0		91 2
Casein												
Corn gluten meal.....												
Egg albumin									18 0			
Gelatin						18 0						
Gliscidin												
Liver			7 4	27 0				42 0				
Soy bean oil meal ..												
Wheat gluten											23 7	
Salts*	4 0	4 0	4 0	4 0	4 0	4 0	4 0	4 0	4 0	4 0	4 0	4 0
Cod liver oil	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0
Wheat germ oil.. . .	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0
Protein	17 35	4 82	4 76	17 43		17 07	15 87	17 87	17 64	18 22	18 86	15 74

* Phillips and Hart (9).

corn gluten meal and wheat gluten were obtained from a local commercial feed market. The egg albumin was prepared in our laboratories. The liver was obtained in a powdered form from Wilson and Company. The soy bean oil meal was a high grade product prepared by the expeller process.

In order to furnish ample amounts of the vitamin B factors a 25 per cent acidulated alcoholic extract of brewers' yeast was evaporated on the dextrin at a level equivalent to 2 per cent of yeast. 0.5 ml. of liver extract was fed individually each day. The liver extract was prepared from an anhydrous liver concentrate manufactured by Wilson and Company. The powdered liver was dissolved in 2 volumes of water. 5 volumes of ethyl ether and 5 volumes of ethyl alcohol were then added and allowed to stand for several hours with frequent shaking. The solvent was then decanted and the precipitate dissolved in water and made up to a volume so that 4 ml. were equivalent to 1 gm. of the original powder. This material was allowed to stand for several hours and then centrifuged, and then again made up to volume. That all of the vitamins were supplied in adequate amounts is evidenced from the excellent growth obtained on Diet 100-P containing 18 per cent of casein.

DISCUSSION

Effect of Added Iron and Copper—Originally it was assumed that the intake of iron and copper from the experimental diets would be adequate for optimal hemoglobin regeneration. The gains in hemoglobin values of anemic rats transferred to Diets 100-P and 101-P, containing 5 and 18 per cent of casein respectively, were virtually parallel. While the animals in the group fed 18 per cent casein practically doubled their weight in 3 weeks, those in the group fed 5 per cent casein just maintained their weight. Both groups required about 20 days for the hemoglobin values to return to normal.

When adequate amounts of iron and copper for optimum hemoglobin formation are added to milk fed to anemic rats, the hemoglobin values will return to normal within a week or 10 days. Since it required about double this time for rats transferred to Diets 100-P and 101-P to regenerate their hemoglobin, we suspected that the iron and copper intake was inadequate. With

the sudden change of regimens the animals may not have immediately accustomed themselves to a solid diet. Consequently, the intake of food during the first few days may have been subnormal, so that the iron and copper consumed were insufficient for optimum hemoglobin regeneration. In order to check this assumption 0.5 mg. of iron and 0.05 mg. of copper were fed individually each day. A solution of ferric pyrophosphate and copper sulfate, together with the liver extract, was fed in a Syracuse type watch-glass. A few of the animals did not readily consume the supplements, in which case they were mixed with a small amount of the

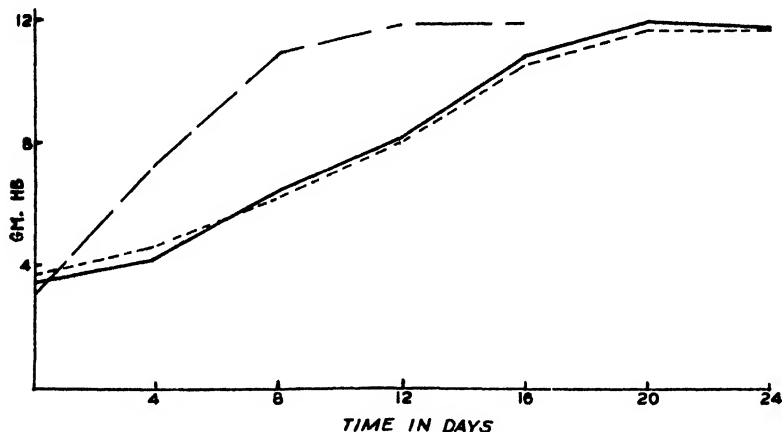


FIG. 1. The effect of Fe and Cu added to experimental diets. The continuous line represents four animals on 18 per cent casein; the broken line, four animals on 5 per cent casein; the dotted line, ten animals on 18 per cent casein plus 0.5 mg. of Fe and 0.05 mg. of Cu daily.

diet. This was eaten within a few hours and the feed was then replenished in the cups in the afternoon.

Feeding additional iron and copper above that contained in the diet greatly accelerated the rate of hemoglobin formation. Fig. 1 shows the effect on the rate of hemoglobin regeneration of feeding additional iron and copper. When 0.5 mg. of iron and 0.05 mg. of copper were fed individually each day to animals on Diet 100-P containing 18 per cent of casein, the hemoglobin values returned to normal within 8 to 12 days; whereas, without the supplements about 20 days were required in order to reach comparable values. Hence, it is evident that the feeding of additional

iron and copper above that contained in the diet is an important factor in the rate of hemoglobin formation. Subsequently, all of the animals, when transferred from the milk diet to the test diet, were fed additional iron and copper individually in the amounts previously mentioned. This procedure eliminated the iron and copper as a variable factor, as the intake was then adequate to permit optimal hemoglobin regeneration even in the most severe cases of anemia.

Quality and Level of Protein—Having eliminated all known factors that might affect the rate of hemoglobin formation, we then carried out studies to ascertain whether the quality and level of proteins influenced hemoglobin regeneration in rats that were made anemic on milk diets. There was considerable variation in the weight of the animals which ranged from 30 to 75 gm. when started on the test diets. However, an inspection of the results failed to reveal any relation between the initial weight of the animal and the rate of hemoglobin formation within a group. When smaller animals were placed on Diet 112-P containing no protein, except for the vitamin addenda, they frequently did not survive the 16 day period. Consequently, it was necessary to select the larger animals for feeding the diets that would not support body weight. Even in some of these cases, a few of the animals were moribund and extremely emaciated at the end of 16 days, especially those on the diet containing no protein.

The results of feeding twelve different diets are summarized in Table II. In each case the number of animals in the group, the average hemoglobin value, and weight at 4 day intervals up to 16 days are recorded. The animals fed the proteins that were adequate for growth generally made the most rapid gains in hemoglobin. Casein, liver, egg albumin, soy bean oil meal, and rat blood were of the same order in inducing hemoglobin formation. At the end of 8 days these groups showed hemoglobin values within the normal range.

Special mention should be made of Diet 123-P in which rat blood was used as a source of protein. Since Pommerenke *et al.* (10) had shown that dog plasma protein given by vein is utilized in body metabolism by the dog and that horse plasma is not similarly utilized, it was decided to use rat blood as a source of protein. It is doubtful that the utilization of blood when fed as

TABLE II
Effect of Various Proteins on Hemoglobin Formation and Body Weight

Protein	Diet No.	No. of animals	Hb, gm. per 100 ml. blood					Weight, gm.				
			Initial	4th day	8th day	12th day	16th day	Initial	4th day	8th day	12th day	16th day
Casein.....	100-P	10	3.19	7.38	11.15	11.96	12.10	48.8	65.1	74.2	84.4	96.4
Low casein	101-P	7	3.43	6.50	9.68	11.48	11.83	49.3	52.0	54.1	55.7	55.7
Liver.....	111-P	10	3.30	7.65	11.87	12.31	12.50	53.8	64.0	77.5	90.0	102.1
Low liver.....	110-P	7	3.31	5.86	8.68	10.12	11.05	55.4	56.0	56.7	58.0	56.1
Egg albumin	118-P	10	3.66	7.25	11.29	11.66	12.01	56.0	70.7	83.7	94.6	105.7
Soy bean oil meal	119-P	10	3.46	7.72	10.84	11.54	11.91	53.5	68.9	78.5	87.2	95.8
Blood.....	123-P	8	3.52	7.99	11.60	12.57	12.13	48.5	54.6	59.4	66.4	72.2
Corn gluten meal.....	121-P	10	3.69	6.69	10.05	10.81	11.25	50.2	53.4	55.3	58.8	60.6
Wheat gluten.....	122-P	10	3.60	6.40	9.37	11.23	11.71	47.7	51.0	52.9	53.2	54.0
Gliadin.....	117-P	7	3.24	6.51	8.15	10.27	10.38	51.6	50.6	49.7	49.3	47.7
Gelatin	120-P	7	3.48	5.95	7.99	9.50	10.40	52.6	51.3	46.1	45.0	42.3
No protein.....	112-P	7	3.41	6.05	8.05	9.13	9.56	59.7	54.4	49.1	46.7	43.8

a normal constituent of the diet differs whether obtained from the same or a foreign species. However, the use of rat blood obviates any question arising on this point in the interpretation of the results. Also, if the regeneration was much more rapid than with other sources of protein, it might be interpreted as a direct transference of dietary hemoglobin to the circulating blood.

Diet 123-P containing rat blood gave a faint salty taste, and because whole blood contains about 400 mg. of sodium chloride per 100 ml. of blood, it was decided to reduce by a corresponding amount the sodium chloride added to the diet. The performance of the animals on this diet was variable; some of the animals gained only a few gm., while others gained as much as 38 gm. in the 16 days. All of the animals showed hemoglobin values of 10.36 gm. per 100 ml. of blood or higher at the end of the 8th day. Thereafter, only slight increases were made in the hemoglobin values.

The animals fed the low casein and liver diets showed average hemoglobin values of 9.68 and 8.68 gm. per 100 ml. of blood respectively on the 8th day, but by the 12th day the values for both groups were above 10 gm. The groups fed corn gluten meal and wheat gluten had average values of 10.05 and 9.37 gm. respectively on the 8th day; by the 16th day the values for the corresponding groups were 11.25 and 11.71 gm. respectively. At the end of the 16 day period the group fed corn gluten had made an average gain of 10.4 gm. and the group fed wheat gluten, 6.3 gm. While the rate of hemoglobin regeneration is slower with the two groups that made only slight gains in weight, it is of interest to note that their hemoglobin values did return to within the normal range by the 12th day.

The groups of animals fed the diets containing gliadin, gelatin, and no protein, which failed to maintain body weight, showed a distinctly slower rate of hemoglobin formation. The average hemoglobin values for the two groups were in the vicinity of 8 gm. per 100 ml. of blood on the 8th day. By the 12th day only the group fed gliadin exceeded 10 gm. The relation of the biological value of the protein to the hemoglobin formation is indicated by the fact that the animals fed gliadin showed less loss in body weight over the 16 day period than did the other two groups, which likewise showed the lowest hemoglobin values up to the 12th day.

The performance of the animals on Diet 112-P, which contained no protein, is of particular interest. From the beginning these animals lost weight, but they gradually rebuilt their hemoglobin. The formation of hemoglobin was, of course, at the expense of body tissues. It is clear from this, as also from the performance of the animals fed gelatin or gliadin, that the requirements for hemoglobin are satisfied before those for growth. It is true that the average hemoglobin value of the group that received no protein was slightly less than 10 gm. per 100 ml. of blood at the end of the 16th day. Apparently the animal may maintain a slightly lower hemoglobin value if it is required to draw from its body tissues for building materials than if the necessary constituents are furnished in the diet.

The hemoglobin values on the 8th day, or the gain in hemoglobin values during the first 8 days, seem to be the most accurate and satisfactory criteria for comparing the effectiveness of the various diets for hemoglobin formation. This assumption is borne out by Fig. 1. Here the differences in the hemoglobin curves which are very striking on the 8th day become smaller on the 12th day and are nil by the 16th day. Even with the diets giving the slowest gains, the hemoglobin values may increase to as much as 10.40 gm. per 100 ml. of blood by the 16th day.

The group of animals fed Diet 111-P, in which the protein is furnished by liver, shows the highest average hemoglobin value on the 8th day. Diet 123-P containing rat blood comes second, and is followed in order by the egg albumin, casein, and soy bean oil meal. Corn gluten meal appears in an intermediate position. Diets 122-P and 101-P containing wheat gluten and 5 per cent casein respectively were slightly less effective in inducing hemoglobin formation. While the values obtained with liver were slightly higher than casein when fed at the higher level, on the lower level casein gave a slightly better response. If liver possessed greater hematopoietic properties than casein or other proteins, there is no reason why this action would not be evinced as readily on the lower level of feeding as on the adequate levels.

The hemoglobin values for the gliadin, gelatin, and no protein diets are distinctly lower on the 8th day than any of the other groups. This is as might be expected if there is any relation between the efficiency of a protein for promoting growth and

hemoglobin formation. These are the only three groups of animals that showed a decline in weight. With the no protein group there was only one animal with a hemoglobin value above 10 gm. per 100 ml. of blood on the 8th day; the lowest value was 6.20 gm.

The average gain in hemoglobin values and standard error of the mean, together with the change in body weight, are recorded in Table III. As body weight is a secondary consideration, only the average gain or loss over the 8 day period is recorded. On this basis of evaluating proteins for hemoglobin regeneration liver

TABLE III
Average Gain in Hemoglobin Values and Change in Weight over 8 Day Period

Protein	Diet No	Hb per 100 ml blood		Weight
		Mean \pm σ^*	Range	
		gm	gm.	gm.
Casein	100-P	7 96 \pm 0 24	6 83- 9 54	25 4
Low casein	101-P	6 25 \pm 0 46	4 51- 7 82	4 8
Liver	111-P	8 57 \pm 0 41	6 02-10 01	23.7
Low liver	110-P	5 36 \pm 0 24	4 52- 6 15	1 3
Egg albumin	118-P	7 64 \pm 0 38	5 93-10 19	27 7
Soy bean oil meal	119-P	7 38 \pm 0 25	6 41- 9 15	25 0
Blood	123-P	8 08 \pm 0 44	6 73-10 12	10 9
Corn gluten meal	121-P	6 36 \pm 0 23	5 23- 7 53	5 1
Wheat gluten	122-P	5 77 \pm 0 17	5 01- 6 61	5 2
Gliadin	117-P	4 91 \pm 0 22	4 05- 5 75	-1 9
Gelatin	120-P	4 52 \pm 0 49	3 23- 6 27	-6 5
No protein	112-P	4 64 \pm 0 38	3 64- 6 26	-10 6

* σ represents the standard error of the mean.

shows the highest value, but is of the same order as rat blood, egg albumin, casein, and soy bean oil meal. Aside from rat blood these proteins have all given good growth. The no protein, gelatin, and gliadin diets were the least effective in inducing hemoglobin regeneration; they were also inadequate to support body weight. The corn gluten meal, wheat gluten, low casein, and low liver diets fall between the two previously mentioned groups. From the standpoint of hemoglobin formation they are distinctly below the proteins that permit good growth, and above those that do not maintain the animal. While the liver fed at

a level to furnish adequate protein again shows the highest value, it is to be emphasized that when fed on the lower level the average gain in hemoglobin is less than with the low casein diet. In view of these results one cannot ascribe to liver hematopoietic properties that are not possessed by casein or other proteins that permit rapid growth, so far as nutritional anemia is concerned.

A comparison of the average gain in hemoglobin values and change in body weights, as recorded in Table III, shows that there is a fair agreement between these factors. With the exception of Diet 123-P, rat blood, those groups that made the greatest gain in body weight likewise made the greatest gain in hemoglobin. This is not strictly true, but it follows that the liver, egg albumin, casein, and soy bean oil meal diets induced gains of about the same order and likewise fell into a superior group for hemoglobin regeneration. The groups fed gliadin, gelatin, and no protein which failed to maintain their weight also made the least rapid gains in hemoglobin. The diets which just maintained the weight of the animals or permitted slight increments were distinctly inferior to the adequate diets in respect to their hematopoietic properties. Inspection of the performance of the individual animals within the groups failed to reveal any such striking relationship between the rates of hemoglobin formation and the change in body weight which occurred between groups.

For further analysis of the data, the gain in hemoglobin value over the first 8 days of the group on 18 per cent of casein is taken as a standard. The significance of the difference between the average gain in hemoglobin of all groups as compared with the casein group has been determined statistically according to Fisher's *t* method (11) for small groups. The results of this statistical study substantiate the previous interpretations. The rate of hemoglobin formation of the group that was fed liver, Diet 111-P, is not significantly higher than in the group fed 18 per cent casein; likewise, the rate of gain in hemoglobin value of the rats fed soy bean oil meal is not significantly lower than that of the group fed casein. The rates of gain of hemoglobin of the groups fed gelatin, gliadin, no protein, wheat gluten, corn gluten meal, low liver, and casein are significantly lower than that for the group fed 18 per cent casein. From the data presented in Table III, giving the average gain in hemoglobin values and the

standard errors, together with the number of observations shown for the corresponding group in Table II, the significance of the difference between the means of any two groups may be calculated.

From the results reported in this paper it is evident that proteins that will permit good growth will allow optimum hemoglobin formation. In searching for an explanation of the apparent discrepancy between the results obtained by Alcock (6) and the observations recorded in this paper, we would draw attention to Fig. 1 showing the effect of feeding iron and copper in addition to that contained in the diet. Here it is seen that the additional iron and copper accelerate hemoglobin regeneration. As previously mentioned, Alcock found that the hemoglobin regeneration was virtually the same with animals fed a tryptophane-deficient diet inadequate for maintenance as for those on a diet to which tryptophane was added and which permitted growth. Alcock apparently depended on the iron and copper intake through the diet to furnish adequate amounts for optimum hemoglobin formation.

While we have not made an exhaustive study of the effect of a deficiency of all the essential amino acids, the results reported in this paper seem to warrant the general hypothesis that, when the dietary protein is either qualitatively or quantitatively inadequate for growth, the rate of hemoglobin regeneration is significantly retarded. Provided adequate amounts of iron and copper are furnished, animals suffering from nutritional anemia will draw upon body proteins for materials to build hemoglobin.

Normal Animals Fed Low Protein Diets—Having established that the rate of hemoglobin regeneration is affected by the level of dietary protein, we considered it of interest to study the effect of feeding low protein diets to normal rats over a long period of time. Bethell (12) reported that the dietary protein is a factor in maintaining normal hemoglobin values during pregnancy in both women and rats. Investigations on the relation of low protein diets to the estrous cycle (13) afforded an opportunity of obtaining at the same time information on hemoglobin.

Non-pregnant female rats were maintained on a diet containing 5 per cent of casein as the sole source of protein, except for the small amount that may have been present in the vitamin addenda. Hemoglobin determinations were made at regular inter-

vals throughout the 70 days the rats were on this regimen. There was no significant change in the hemoglobin values after the animals were transferred from a stock diet to a low protein diet, normal hemoglobin values being maintained throughout this 70 day period. Gliadin was then added to the diet of one group of animals and gelatin added to the diet of another group. The animals receiving gliadin increased in weight and resumed normal estrous cycles, while the group fed gelatin failed to respond in either respect. This alteration in the diets was without significant effect on the hemoglobin in both groups of animals. From these observations it is evident that non-pregnant rats fed low protein diets for long periods of time will maintain normal hemoglobin, provided adequate amounts of iron and copper are fed.

SUMMARY

Studies were made of the effect of nine different proteins on the rate of hemoglobin regeneration in nutritional anemia.

The proteins of liver, casein, egg albumin, and soy bean oil meal are consistently effective in building hemoglobin. When the intake of iron and copper is adequate to permit hemoglobin formation at the maximum rate, no special hematopoietic property in addition to their protein content can be ascribed to any of these materials. There is no evidence that liver is more effective for hemoglobin formation than are proteins of good quality. Rat blood permitted excellent hemoglobin regeneration, but the growth response was somewhat inconsistent.

Corn gluten meal and wheat gluten are poorly utilized by the animal for both the formation of hemoglobin and growth. Liver and casein fed at a level to furnish 4.8 per cent of protein permit hemoglobin regeneration at approximately the same rate as corn gluten meal or wheat gluten fed at a level to furnish about 18 per cent of protein. Animals fed gliadin or gelatin, which are inadequate for growth, rebuild their hemoglobin less rapidly than when fed a protein that permits growth.

It is clear that the formation and maintenance of normal hemoglobin values is more vital than growth and that the animal will utilize its body proteins for this purpose, provided adequate amounts of iron and copper are fed. When the dietary protein

is either qualitatively or quantitatively inadequate for growth, the rate of hemoglobin regeneration is significantly retarded.

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CHEMICAL STUDIES ON THE NEUROPROTEINS

I. THE AMINO ACID COMPOSITION OF VARIOUS MAMMALIAN BRAIN PROTEINS

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Chemical studies of the nervous system have dealt largely with its lipoidal constituents. The more labile proteins, which are present in a smaller proportion, have been the subject of relatively few investigations, although their fundamental importance for neurophysiology and neuropathology cannot be doubted. In order to prepare a proper foundation for our contemplated investigation of the proteins and protein fractions obtained from normal and pathological human brains, it was of interest to ascertain the amino acid composition of the brain proteins from different mammals. (The need for such a study has recently been discussed at length by Kaplansky (1) and, therefore, does not have to be repeated here.)

Some of the analyses were carried out on the whole protein of the brain, some were carried out on a large fraction thereof. This was deemed advisable in a comparative chemical investigation because fractionation of the protein complex would yield results which might be complicated by unavoidable variations in the extraction procedures (*cf.* (2) for a detailed discussion of this point).

EXPERIMENTAL

Preparation of Human, Monkey, Beef, and Sheep Brain Proteins—The fresh brains (1000 to 7000 gm.), obtained less than 2 hours after death, were cooled to 0° and the meninges and larger blood vessels were removed by dissection. The tissue was then cut into thin slices and carefully washed with cold water until free

from blood. The washed brain was placed in a freezing mixture overnight and then allowed to thaw to 4°. The thawed material was ground six times in an electric meat grinder and suspended in 5 times its weight of ice water. Chloroform was added at this point. The suspension was allowed to stand at 0° for 48 hours and the precipitate removed by centrifugation. The supernatant liquid was filtered in the cold through soft paper and the protein precipitated by the addition of 20 per cent HPO₃ to pH 3.8. The precipitate was centrifuged off, washed with dilute HPO₃, and dried with acetone. The resulting dry powder was exhaustively extracted with a hot benzene-alcohol solution (benzene 95, absolute alcohol 5), hot alcohol, and ether. The resulting white powder which required about 3 weeks to prepare, was dried in the

TABLE I
Chemical Constitution of Brain Proteins

Brain protein	Nitro- gen	Histi- dine	Lysine	Arginine	Cystine	Trypto- phane	Tyrosine
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Human	13.4	2.3	4.4	5.0	1.5	1.3	3.9
Monkey	12.8	2.1	4.8	5.4	1.5	1.1	3.8
Beef	13.9	2.2	4.0	5.0	1.4	1.3	3.8
Sheep	12.5	2.3	4.1	5.0	1.2	1.1	3.6
Rat	14.6	2.8	4.0	4.9	1.4	1.3	4.2
Guinea pig	14.8	2.4	4.2	5.2	1.4	1.0	4.1

desiccator and in the oven at 110° to constant weight. This material was used for analysis.

Preparation of Rat and Guinea Pig Brain Proteins—On account of the relatively small amount of this material available at any one time, the brain slices from these animals, which were killed by decapitation, were stored in acetone until a sufficient quantity had accumulated. The acetone-hardened brains were ground and extracted with acetone, benzene, alcohol, and ether as described above. The resulting powder, after drying to constant weight, was used for the analysis.

Analyses of Brain Proteins—Tyrosine and tryptophane were estimated by the Folin-Marenzi (3) method and cystine by the Folin photometric method (4). The determinations of the basic amino acids were carried out by the procedure previously de-

scribed (5), except that the value given for histidine was obtained by Conrad and Berg's modification of the Kapeller-Adler method (6). In each case, however, the presence of histidine was proved by the isolation of the difluorinate. Nitrogen was estimated by the Kjeldahl method, with copper and selenium as catalysts. The analytical results, based upon two or more determinations, are summarized in Table I. It will be noticed that the amino acid composition of the entire protein fraction of the rat and guinea pig brains did not differ significantly from the "soluble" brain protein complex prepared from the larger animals.

DISCUSSION

These results are in agreement with those of Kaplansky (1), who found that the entire brain protein complex of different verte-

TABLE II
Molecular Ratios of Amino Acids

Brain protein	Molecular ratio of									
	Cystine to Tryptophane		to Histidine		to Tyrosine		to Lysine		to Arginine	
Human	21	to	21	to	49	to	72	to	100	to 96
Monkey	19	"	16	"	41	"	64	"	100	" 95
Beef	23	"	23	"	52	"	77	"	100	" 105
Sheep	18	"	18	"	53	"	71	"	100	" 103
Rat	21	"	23	"	66	"	85	"	100	" 103
Guinea pig	20	"	17	"	54	"	78	"	100	" 104

brates yielded approximately the same amounts of tyrosine, tryptophane, cystine, and probably phenylalanine. It will be seen from Tables I and II that the amounts of histidine, lysine, arginine, cystine, tryptophane, and tyrosine obtained from the six mammalian brain proteins were quite similar. In fact, the constancy of these amino acids was greater than that shown by the total nitrogen of the different preparations. This indicates that these brain protein preparations probably vary in the composition of some of their amino acids for which analyses were not carried out. Such a variation in the composition of the mammalian brain proteins was expected, for we had previously found (7) differences in the immunological properties of the nucleoproteins from cattle and pig brains, while Kaplansky's coworkers have

reported differences in the alanine (8) and arginine (9) content of mammalian and avian brain proteins.

The molecular ratios of these amino acids are summarized in Table II. It will be seen that although the amounts of the amino acids vary somewhat among the different animals, the ratios remain quite constant. Attention is especially called to the molecular ratios of lysine to arginine and to the somewhat lower ratios found in the human and monkey brain proteins.

These experiments illustrate again the relative constancy of the amino acid composition of tissue proteins. The importance of such findings has been discussed previously (2, 10).

SUMMARY

Proteins have been prepared from human, monkey, beef, sheep, rat, and guinea pig brains. These proteins were analyzed for nitrogen and six amino acids, the following average values being obtained: nitrogen 13.6 per cent, histidine 2.4 per cent, lysine 4.3 per cent, arginine 5.1 per cent, cystine 1.4 per cent, tryptophane 1.2 per cent, and tyrosine 3.9 per cent. The results indicate that all six brain proteins contain approximately the same relative proportions of these amino acids although they may vary in absolute amounts. Attention is called to the remarkable constancy of the molecular ratio of lysine to arginine in four of the proteins analyzed and to the somewhat lower ratios found in the human and monkey brain proteins.

I am indebted to Dr. N. Kopeloff for his kindness in furnishing me with an ample number of monkey and guinea pig brains and to Miss B. Kassell for carrying out the cystine determinations.

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VARIATIONS IN LIPEMIA OF NORMAL SUBJECTS*

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To relate the rise and fall in serum lipids of diseased patients with changes in clinical condition, it has been found essential to define the constancy of the lipid fractions in the blood serum of normal men and women. Over 2 years ago when this project was started Sperry's recent article had not been published (21), Gardner and Gainsborough (3) had limited their prolonged study to one person, and Okey and coworkers (14, 15) had restricted their research to the female sex. Moreover, these investigators determined cholesterol but not the other lipid fractions in blood or blood serum. In this investigation the serum cholesterol, phosphatides, fatty acids, and generally the total protein, albumin, and globulin, of six women and four men between 24 and 45 years in age have been determined at intervals throughout a total length of time of 3 months to 4 years. The intervals between blood studies were irregular in four individuals, were of 2 weeks duration throughout 3 months in two males and two females, and then were prolonged to approximately 1 month in one male and two females. Such timing of the blood studies has permitted investigation of the level of serum lipids in relation to the season of the year, to the menstrual cycle of women, and to the constancy of lipemia after intervals of time as prolonged as $1\frac{1}{2}$ years in two subjects, $2\frac{1}{2}$ years in one male, and 4 years in one female.

Methods

At the time that each blood sample was taken the individual was in a postabsorptive state and in apparently normal health.

* This investigation was aided by a grant from the Knight Fund of Yale University School of Medicine.

Blood serum was analyzed by methods previously described for cholesterol (10), phosphatide phosphorus (7, 10), total serum proteins, albumin, globulin (2), and titrated fatty acids (8). Titrated fatty acids differ from the previously mentioned total fatty acids in that all the phosphatide fatty acids are thought to be included in the titrated fatty acids. Data concerning this point are to be published (4). In 100 consecutive determinations of serum lipids it has been found that the correlation between one duplicate and the other duplicate cholesterol is 0.978, or that given one of two duplicates the other can be estimated within a probable error of ± 10.84 mg. per cent. The correlation between duplicate fatty acid determinations is 0.987 and the probable error of estimate ± 0.49 milli-equivalents. Lipoid phosphorus determinations have a correlation of 0.983, and a probable error of estimate of one of a pair of duplicates from the other is ± 0.29 mg. per cent.

Data

In Figs. 1, 2, and 3 are given the serum cholesterol, lipid phosphorus, titrated fatty acids, total protein, and body weight of three normal male subjects. The subjects E. G. and W. B. were studied for more than 1 year. Solid lines connect the points representing the findings during the 1st year and dotted lines during the 2nd year. The curves are superimposed so that any seasonable trend from year to year may be recognizable. Figs. 4, 5, and 6 represent the findings on three female subjects, the first of whom was studied for 4 years and the second for 1 complete year. The dates of the beginning of each menstrual period are marked.

In Table I, the minimum and maximum figures and the mean of all the values on each subject are given. These values represent the lowest and highest for any of the six serum constituents regardless of the date. For example, the minimum cholesterol may have been obtained at some other time than the minimum lipid phosphorus. The differences between the minimum and maximum figures for each constituent are expressed as the per cent deviation from the mean of all values. Thus the cholesterol of E. G. changed 66 mg. per cent from a low of 173 to a high of 239 mg. per 100 cc. or 31 per cent of 213 mg. per 100 cc.

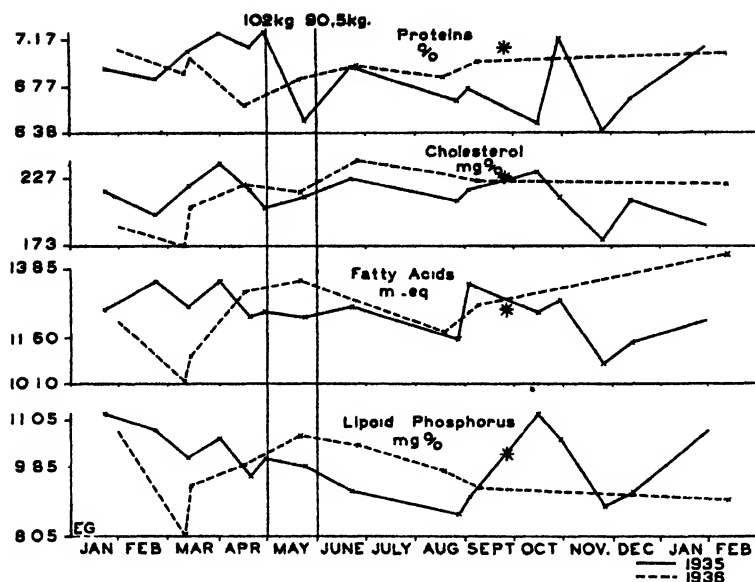


FIG. 1. E. G., male; weight 98 to 104 kilos, except after dieting as indicated on graph. The large asterisks represent findings in September, 1934.

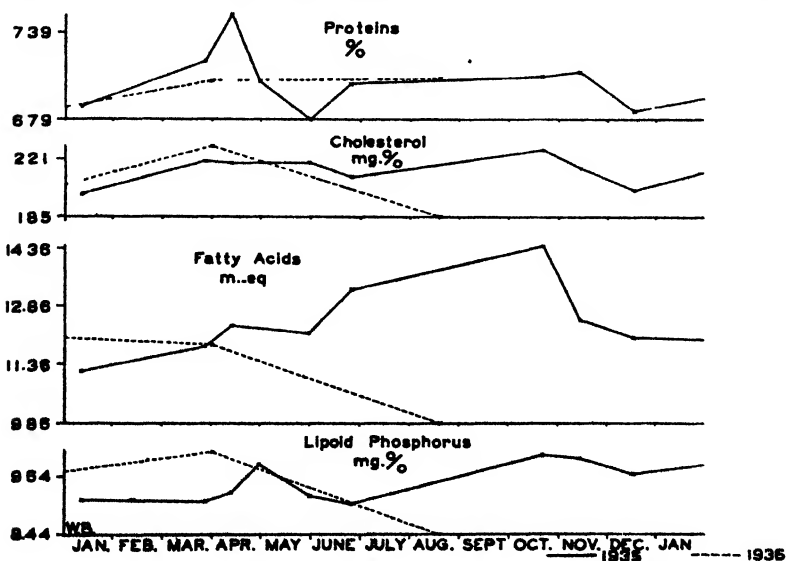


FIG. 2. W. B., male; weight 65 to 69 kilos

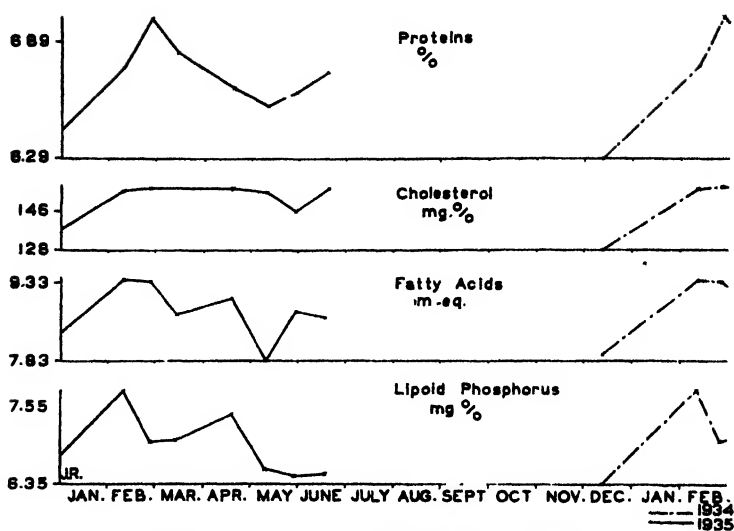


FIG. 3. J. R., male; weight 78 to 79.2 kilos

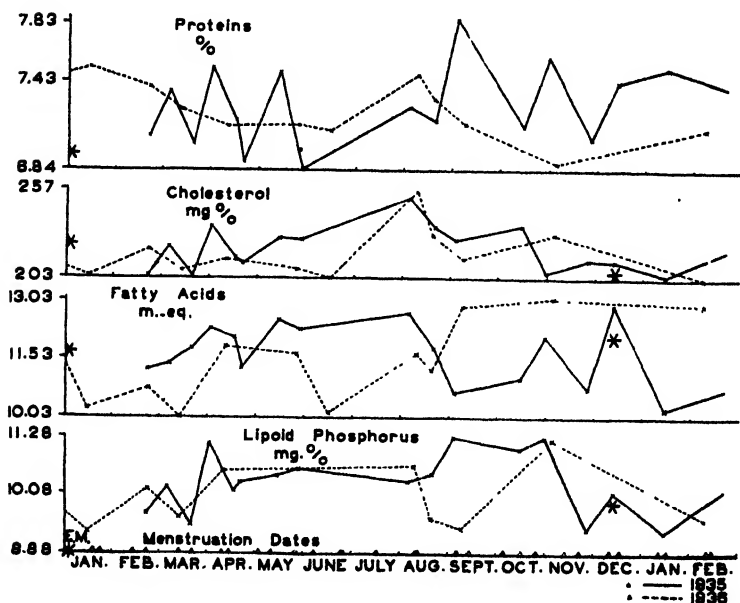


FIG. 4. E. M., female; weight 51.8 to 54.7 kilos. The * represent findings in December, 1932, and January, 1933.

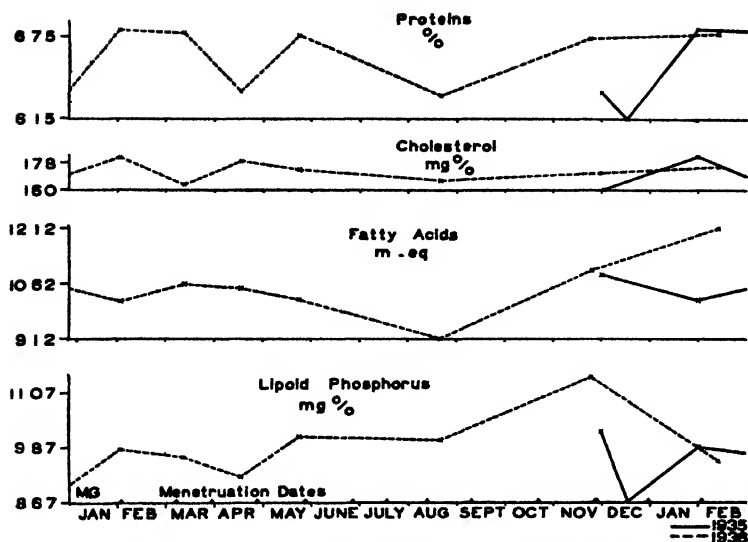


FIG. 5. M. G., female; weight 61.8 to 63.6 kilos

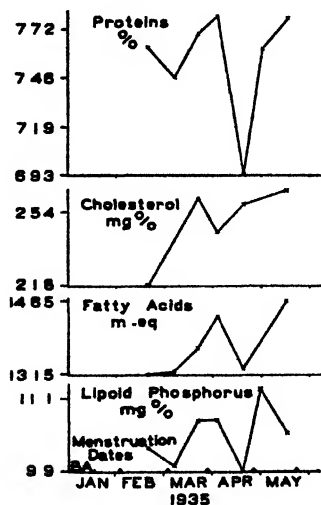


FIG. 6. B. A., female; weight 59.5 to 60.6 kilos

TABLE I
Serum Lipids and Proteins of Normal Subjects

Subject	Sex	Cholesterol				Lipid phosphorus				Titrated fatty acids				Protein				Albumin				Globulin																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																													
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cent	per cent	per cent	per cent	per cent

Results

In Table I it is demonstrated that there is a wide range in the serum cholesterol, lipid phosphorus, titrated fatty acids, and total protein of any one individual. The cholesterol of E. G. (male) varied from a minimum of 173 to a maximum of 239, 66 mg. per 100 cc., a difference of 31 per cent, while that of E. M. (female) varied from 203 to 257, 54 mg. per 100 cc. or 24 per cent. These were the greatest absolute differences observed in the ten subjects studied and were obtained on the two persons who were followed for the longest periods of time, 2½ and 4 years respectively.

The titrated fatty acids of these two subjects varied 3.14 and 2.86 milli-equivalents or 25 and 24 per cent. These differences were exceeded by only one subject W. B. (male) whose fatty acids changed 37 per cent, that is 4.55 milli-equivalents, which would represent 122 mg per cent if expressed as tripalmitin. Again the greatest changes in lipid phosphorus occurred in the two subjects studied longest: the lipid phosphorus of E. G. varied from 9.20 to 11.22 mg. per 100 cc. or 21 per cent, and that of E. M. from 8.88 to 11.28 mg. per 100 cc. or 23 per cent. Percentage changes in total serum proteins were not as great as in the lipids, the greatest changes being 13 and 14 per cent and the greatest absolute change from 6.84 to 7.87 per cent. Percentage differences in serum globulin exceeded those of serum albumin but marked alterations in globulin have already been observed by Peters and Eisenman (17).

In the figures it is obvious that frequently the three lipid fractions increased or decreased simultaneously. This relation has its exceptions and is qualitative rather than quantitative.

DISCUSSION

Whether differences in serum lipids merely reflect changes in blood volume was a question raised by a previous investigation in which it was shown that during short intervals of time the capillaries are impermeable to cholesterol, phosphatides, and certain fatty acids (11). If these alterations in serum lipids are related to hemoconcentration or hemodilution the total protein should be affected in the same manner as the lipids. However, in the six figures it is obvious that the lipids and proteins vary inde-

pendently. For example, the proteins of E. G. were almost identical in March and June, 1936, when the cholesterol rose from 173 to 239 mg. per cent, and the fatty acids from 10.14 to 14.08 milli-equivalents. The cholesterol of W. B. fell from 227 to 185 mg. per cent and the fatty acids from 14.41 to 9.86 milli-equivalents from October, 1935, until August, 1936, although the total serum proteins were 7.08 per cent at the times of both blood studies. In Fig. 6 the independence of cholesterol and proteins is demonstrated. In Fig. 4 it may be seen that the proteins were the same in January and in August, 1936, although the cholesterol made a low and a high, and again that the proteins were identical in August, 1935, and March, 1936, when the fatty acids varied.

Any relation between the variations in serum lipids and proteins and changes in body weight or food intake seems improbable. All the subjects studied were well nourished and on a balanced diet which varied little during summer and winter. Exclusive of E. G., the weight of each subject was constant within 6 per cent or less than 6 pounds for each individual, except W. B. who lost 9 pounds. By dieting, E. G., between April 1 and May 1, 1935, reduced his weight 27 pounds, about 12 per cent. During this month his serum lipids and proteins varied no more than at other times throughout the course of the study. In about 2½ months he regained these pounds and during the rest of the 30 month period his weight varied only about 8 per cent.

That changes in serum lipids and especially in the cholesterol of females can be related directly to the menstrual cycle is questionable because the lipids and cholesterols of males are subject to as great variations as those of females. The cholesterols of the four males in Table I showed differences of 31, 20, 19, and 18 per cent, while those of the six females changed 24, 19, 13, 11, 9, and 1 per cent. In data recently published by Sperry (21) these same large divergences in the serum cholesterols of males are apparent. The male subjects, Nos. 6, 62, and 85 of Sperry's report, showed differences between the minimum and maximum cholesterols of 16.1, 19.6, and 24.6 per cent, while the females, Subjects 69, 73, and 86, who exhibited the widest variation in serum cholesterol, had differences of 19.4, 12.2, and 18.8 per cent. Similarly in the present investigation the fatty acids of the males varied 37, 25, 17, and 16 per cent, while those of the females

differed by 24, 17, 11, 10, 7, and 5 per cent. Moreover, in Figs. 4, 5, and 6 it can be seen that the high and low cholesterols bear no relation to the dates of menses which are included in these figures. E. M. had a cholesterol of 205 and fatty acids of 10.2 milli-equivalents on January 14, 1936, 3 days before the onset of menstruation and a cholesterol of 252 and fatty acids of 12.7 milli-equivalents on August 6, 1935, 4 days before menstruation began. M. G. showed a variation in cholesterol of only 22 mg. per cent and in fatty acids of 1.8 milli-equivalents, although the blood samples were taken just before, just after, and half way between menses. Similarly, with the exclusion of the first cholesterol of B. A. this serum constituent varied only 24 mg. per cent and fatty acids varied only 1.5 milli-equivalents, although blood samples were taken 10, 8, and 3 days before, and 3 and 8 days after menstruation. The early observations of Okey and Boyden (14) on the relation of blood cholesterol to the menstrual cycle showed an amazing irregularity in the cholesterol. Okey did not relate definitely these irregularities to the menstrual cycle but did state that a fall in blood cholesterol "during or within a few days of the menstrual period . . . was usually preceded or followed by blood cholesterol levels higher than the average for the individual concerned." The variability in cholesterol was not so great in the later investigation of Okey and Stewart (15), when a digitonin method had replaced the colorimetric procedure for the determination of cholesterol. Moreover, the relation of cholesterol to the menstrual cycle is not precise in this later study of Okey in which the more exact digitonin method was used.

No definite seasonal pattern of the serum lipids can be formulated from the six figures. In this they differ from the observations of Petersen (18). In Figs. 1 and 4 which show the lipids and proteins of the two persons followed longest, the fatty acids, proteins, and phosphatides exhibit a variable course throughout 2 successive years. This is also exemplified in Fig. 2, while the other three subjects were not studied long enough for any conclusion to be drawn. The course of cholesterol seems to be somewhat reproducible from year to year in Figs. 1 and 4, for E. G. had his lowest cholesterol in February, 1935, and March, 1936, and E. M. had her highest cholesterol in August, 1935 and 1936. On the other hand, M. G. showed very little change in cholesterol

throughout the course of 1 year. These data are not typical of any precise seasonal pattern but would suggest peculiar individual patterns.

In relating changes in clinical condition of pathological subjects with serum lipids, it must be remembered that in normal subjects the lipids tend to vary up and down from the average, while in patients with disease an increase or decrease if continued long enough should be significant. For example, it has been shown that as the malnutrition of patients was alleviated, the serum cholesterol rose 32 to 101 mg. per cent during intervals of time of 2 to 10 weeks (9). The reduction in serum cholesterol after the administration of thyroid extract to myxedema patients is marked and unequivocal (6, 13). Similarly, in simple hyperthyroidism the administration of Lugol's solution and thyroidectomy are attended by a definite increase in cholesterolemia (5). In diabetic acidosis the fall in serum fatty acids of 200 per cent in four of fourteen cases and in cholesterol of as much as 60 per cent occurs in 18 to 48 hours (12). Such great changes or prolonged variations in lipemia are in sharp contrast to the behavior of the serum lipids of the normal subjects.

The large variations in serum lipids of the subjects studied in this investigation agree with data already published. Sperry (21) has reported the constancy of the cholesterol of 16 males and 9 females and found maximum changes of 24.6, 19.6, and 19.4 per cent. Gardner and Gainsborough (3) determined the total, free, and esterified cholesterol of one subject at intervals during 3 years and found a variation of 56 mg. per 100 cc. or of 28 per cent (calculated in the same manner as the above data) in total cholesterol. The variations in free cholesterol were from 51 to 78 mg. per cent and in the esterified fraction from 99 to 159 mg. per cent. Okey and coworkers (14, 15) in two papers which have already been discussed found by a colorimetric procedure a wide range in the cholesterol of female subjects, and by a digitonin method the cholesterol of four females on constant low cholesterol diets changed from 152 to 201, from 137 to 177, from 147 to 190, from 128 to 168 mg. per cent. Boyd (1) and Poutchinsky and Glouhenky (19) have reported large diurnal variations in serum lipids within a period of only 24 hours. The cholesterol of a single individual is therefore not constant, a fact which is consist-

ent with the wide range of normal cholesterol values which has been fully discussed by Page, Kirk, Lewis, Thompson, and Van Slyke (16). The constantly changing level of serum lipids may be an indication of the continual activity of fat metabolism which was demonstrated recently by Schoenheimer and Rittenberg (20).

SUMMARY

Serum lipids of four males and six females have been examined at intervals for periods of time of 3 months to 4 years in duration. The minimum and maximum serum cholesterol varied by as much as 31 per cent, lipid phosphorus by 23 per cent, titrated fatty acids by 37 per cent, and proteins by 14 per cent.

Differences in lipids were not related to changes in hemoglobin concentration when serum proteins were employed as a criterion of blood volume.

Variations in lipids were not related to slight changes in body weight.

In females the course of serum cholesterol could not be correlated with the menstrual cycle, and, moreover, changes in the cholesterol of males exceeded those in females.

No definite trend in serum lipids in relation to the season of the year could be traced from person to person.

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**PROCEEDINGS OF THE AMERICAN SOCIETY OF
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On the Equilibrium between Oxygen and Respiratory Pigments.

BY ELLIOTT T. ADAMS. *From the Department of Biological Chemistry, Tufts College Medical School, Boston*

Adair's equation may be written in the general form

$$z = \frac{\sum_{q=1}^n \binom{n-1}{q-1} K_q x^q}{1 + \sum_{q=1}^n \binom{n}{q} K_q x^q} \quad (1)$$

where x denotes the oxygen pressure, z the degree of saturation, K_q equilibrium constants, and n the number of oxygen molecules with which 1 molecule of a respiratory pigment combines. Let ν be defined by the equation

$$\nu = \frac{d \log \frac{z}{1-z}}{d \log x} = \frac{x}{z(1-z)} \frac{dz}{dx} \quad (2)$$

Upon substituting Equation 1 and its first derivative in this equation, we find

$$\nu = n \frac{\sum_{q=1}^n q \binom{n-1}{q-1} K_q x^{q-1} - z \sum_{q=1}^n n \binom{n-1}{q-1} K_q x^{q-1}}{\sum_{q=1}^n n \binom{n-1}{q-1} K_q x^{q-1} - z \sum_{q=1}^n n \binom{n-1}{q-1} K_q x^{q-1}} \quad (3)$$

where z is given by Equation 1. Bearing in mind that all the K_q must be zero or positive, we see from Equation 3 that if Equation 1 is valid,

$$\nu \leq n. \quad (4)$$

If we suppose we have a mixture of respiratory pigments, each of which combines with oxygen according to Equation 1, and if $n_{\max.}$ denotes the largest value of n encountered in the mixture, it can be shown that

$$\nu \leq n_{\max.} \quad (5)$$

an inequality which we may consider to include Equation 4 as a special case.

A comparison of the work of Wastl and Leiner¹ with the work of Svedberg and Hedenius indicates that when we deal with some avian bloods Equation 5 is not obeyed, ν exceeding n_{\max} , when z is sufficiently large. There is evidence, therefore, that the oxygen dissociation curves of certain bloods cannot be represented by Adair's equation, or even by the more general equation obtained when a mixture of respiratory pigments is assumed to be present.

The Determination of Blood Creatine with 3,5-Dinitrobenzoic Acid As a Color Reagent. BY JEROME E. ANDES. *From the Laboratory of Clinical Pathology, West Virginia University Medical School, Morgantown*

A method has been worked out for the determination of creatine in blood, with 3,5-dinitrobenzoic acid as color reagent. The creatinine is determined before and after autoclaving the tungstic acid filtrate (color reagent used as described by Langley and Evans), and the creatine obtained from the difference. A noteworthy point is the manner of protein precipitation (the reagents are added in *reverse order*), since the filtrate so obtained remains *entirely colorless* when autoclaved in acid solution.

A series of determinations of creatine (in pure solution and added to blood) was made by the new method, and the results were compared with those obtained by simultaneous determinations with picric acid. Pure creatine solutions and blood containing added creatine showed almost identical results with both methods. Creatine added to blood was almost completely recovered. Creatine determinations on the blood of a series of normal adults likewise showed fair agreement (an average of 3.3 mg. per cent by the 3,5-dinitrobenzoic acid method and 3.1 mg. by the picric acid method). This agreement of the two methods is evidence in favor of the chromogenic material in the blood actually being creatine. The low values in both cases are attributed to the method of protein precipitation.

The determination of blood creatine by the new method seems quite satisfactory, but it has certain disadvantages that make one hesitate as yet to suggest it in preference to the picric acid procedure.

¹ Wastl. H.. and Leiner, G., *Arch. ges. Physiol.*, **227**, 367, 421, 460 (1931).

The Solubility of Amino Acids in Concentrated Sulfuric Acid.

BY JAMES C. ANDREWS. *From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

Amino acids, as a rule, are highly soluble in concentrated sulfuric acid and may be recovered unchanged from such solution. Not only can the highly water-soluble amino acids be thus dissolved but cystine and tyrosine will dissolve in concentrated sulfuric acid to form solutions containing more than 25 per cent of the amino acid. Such solutions of *l*-cystine are stable for some weeks at 38°, during which the cystine can be recovered with its optical activity practically unchanged. The optical activity of *l*-cystine dissolved in concentrated sulfuric acid was also determined. $[\alpha]_D^{25} = -287^\circ$ for a sample which under standard conditions in hydrochloric acid $[\alpha]_D^{25} = -206^\circ$.

In sulfuric acid tyrosine readily forms the disulfonate. This compound has been prepared both as the free acid and as the barium salt.

Fluorine Content of Enamel and Dentin of Sound and Carious Teeth. BY W. D. ARMSTRONG. *From the Laboratory of Physiological Chemistry, University of Minnesota, Minneapolis*

The mean fluorine content of the enamel of twenty-nine sound teeth was 0.0112 per cent and that of fourteen carious teeth was 0.0067 per cent. The enamel of ten sound teeth from one mouth contained 0.0096 to 0.0117 per cent fluorine and that of each of two carious teeth contained 0.0066 and 0.0060 per cent fluorine. The range of fluorine content of the enamel of twelve sound teeth from another mouth was 0.0098 to 0.0130 per cent. The enamel of two carious teeth from the same case contained 0.0056 and 0.0078 per cent fluorine. The mean fluorine content of the mixed enamel of seven sound teeth from a third case was 0.0160 per cent, and the enamel of four carious teeth from the same mouth, separately analyzed, contained 0.0058 to 0.0087 per cent fluorine. In a study of the composition of enamel and dentin this is the first possible significant difference in composition found between sound and carious teeth. The mean fluorine content of the dentin of the sound teeth of the last case was 0.0204 per cent, and that of the carious teeth was not significantly lower, being

0.0184 per cent. The fluorine content of the enamel and dentin of mottled teeth is being investigated.

The Oxidation-Reduction Potentials of Spirographis Hemin and of Some of Its Hemochromogens. By E. S. GUZMAN BARRON.
From the Lasker Foundation for Medical Research and the Department of Medicine of the University of Chicago, Chicago

Warburg and his coworkers presented spectroscopic evidence that the heme nucleus of cellular hemochromogens (Warburg's *Sauerstoffübertragendes Ferment*) is not of the blood hemin type but of the pheohemin type, the spectrum of CO-*Spirographis* hemoglobin resembling that of the CO compound of the *Sauerstoffübertragendes Ferment*. *Spirographis* hemin, according to H. Fischer and Seeman, is the FeCl compound of 1,3,5,8-tetramethyl-4-vinyl-2-formylporphin-6,7-dipropionic acid, while in blood hemin there is no formyl group but instead a vinyl group. To study the relation between chemical structure and free energy of hemins, the oxidation-reduction potentials of *Spirographis* hemin were investigated by potentiometric titrations at 30°.

Spirographis hemin is, like blood hemin, an electromotively active, reversible oxidation-reduction system, the E_h values agreeing with those calculated from the equation

$$E_h = E'_0 - \frac{RT}{nF} \ln \frac{(\text{Fe}^{++} \text{ hemin})}{(\text{Fe}^{+++} \text{ hemin})}$$

where $n = 1$. The E'_0 of *Spirographis* hemin at pH 9.63 is -0.237 volt, that of blood hemin is -0.292 volt. The E'_0 values of the following hemochromogens of *Spirographis* hemin are also more positive than those of the corresponding hemochromogens from blood hemin. At pH 9.63 E'_0 of cyanide *Spirographis* hemochromogen is -0.113 volt; of blood hemin, -0.182 volt. E'_0 of pilocarpine *Spirographis* hemochromogen is -0.068 volt; of blood hemin, -0.170 volt. E'_0 of α -picoline *Spirographis* hemochromogen is -0.016 volt; of blood hemin, -0.033 volt. The replacement of a vinyl group in blood hemin by a formyl group in *Spirographis* hemin is also accompanied by a shift toward the red end of the spectrum by about 35 Å. of the α bands of all of the ferro forms of *Spirographis* hemochromogens as compared with the ferrohemochromogens from blood hemin.

A Rapid Method for Quantitative Determination of Tryptophane.

By ROBERT W. BATES. *From the Carnegie Institution of Washington, Station for Experimental Evolution, Cold Spring Harbor*

In the method of May and Rose maximum development of the blue-colored compound, formed by union of the *p*-dimethylamino-benzaldehyde with the indole ring in the presence of 6 N HCl, is obtained only after 2 days in an incubator or even longer at room temperature.

By addition of a suitable accelerator (oxidizing agent) and by developing the colored compound in 10 to 11 N HCl, maximum color formation is obtained at room temperature in 5 to 10 minutes and the whole determination completed in 30 minutes. Casein unknowns can be determined as accurately as one can match the colors, with maximum errors of about 2 to 3 per cent.

The most rapid procedure is as follows: Dissolve a protein sample (e.g. 50 mg. of casein) in 2 ml. of 0.1 N NaOH in a small beaker. At once add 0.5 ml. of 5 per cent *p*-dimethylaminobenzaldehyde solution, 0.2 ml. (2 mg.) of NaNO₂ solution, and 25 ml. of concentrated HCl. Cover and allow to stand 15 minutes for full color development. Then pour into a 50 ml. volumetric flask and dilute to volume with water. Let stand 15 minutes to cool before comparing colorimetrically.

A less rapid method, employing only the accelerator, is as follows: Mix protein, aldehyde, NaNO₂, water, and HCl all together, adding the HCl last. Place in an incubator at 37–40° and remove and compare the samples at 2 hour intervals to obtain the maximum. This is the method of May and Rose, with the addition of the accelerator. The time to reach the maximum color intensity is inversely proportional to the amount of NaNO₂ (e.g. 2 mg. of NaNO₂ per 50 ml. require 4 hours; 4 mg. of NaNO₂ per 50 ml. require only 2 hours).

The Dietary Production of Parathyroid Hypertrophy in Rabbits.

By EMIL J. BAUMANN AND DAVID B. SPRINSON. *From the Laboratory Division, Montefiore Hospital, New York*

Rabbits fed on a diet of carrots and oats develop parathyroid hypertrophy. The two external parathyroids may increase from a normal weight of about 10 mg. to over 40 mg. Microscopically

the cells and nuclei are enlarged about 50 per cent and contain more lipids than the normal parathyroid. The color of the gland changes from pale pink to a salmon-pink. The carrot and oat diet has a Ca to P ratio of 0.5, whereas in our stock diet of alfalfa and oats and occasional greens the ratio is about 4.

The serum Ca of these rabbits is normal. The inorganic PO_4 is usually at the lower limit of normality or below. Within a week, rabbits on a carrot and oat diet show an increased amount of circulating parathormone by Hamilton's tests, and this continues even after 6 to 8 months on this diet. The low serum phosphatase of normal adults is decreased to almost zero by this diet.

A Polyhydroxy Acid from the Sweet Pepper; a Correction. BY
EMIL J. BAUMANN, DAVID B. SPRINSON, AND NANNETTE
METZGER. *From the Laboratory Division, Montefiore Hospital,
New York*

The polyhydroxy acid which we reported having isolated from the press-juice of the sweet pepper (*Capsicum annuum*)² has proved to be dimethyl citrate. Our error was due to the fact that dimethyl citrate crystallizes with 1 molecule of water of crystallization which is not removed by prolonged drying over P_2O_5 above 100° at 10 to 15 mm. pressure. Proof that the substance was dimethyl citrate was obtained by preparation of the trimethyl ester and the methyl ether of trimethyl citrate. Since citric acid forms a dimethyl ester fairly readily and methyl alcohol was used in the process of isolation, it is probable that the ester does not exist in the press-juice of the sweet pepper but was formed in the process of isolation.

Methionine As the Limiting Nutritive Factor of Arachin. BY
ELIOT F. BEACH AND ABRAHAM WHITE. *From the Laboratory
of Physiological Chemistry, Yale University School of Medicine,
New Haven*

The observations of previous investigators that arachin is incapable of supplying the protein requirement for normal growth in the rat have been confirmed. Analyses indicate that the

² Baumann, E. J., Sprinson, D. B., and Metzger, N., *Proc. Am. Soc. Biol. Chem.*, **8**, v (1935) (*J. Biol. Chem.*, **109** (1935)).

methionine content of arachin is low (0.5 per cent). Experiments were therefore designed to determine whether methionine would stimulate growth in animals stunted by a diet in which arachin served as the source of protein. Rats (21 days old) were fed a diet having the following composition: arachin 15, starch 55, Crisco 21, salt mixture (Osborne and Mendel³) 4, and cod liver oil 5 per cent. Each animal received an adequate daily supplement of the vitamin B complex. In fifteen animals on this diet, the average daily growth was 0.23 gm. The addition of 450 mg. of *dl*-methionine to each 100 gm. of the diet resulted in immediate growth stimulation. In a group of twelve animals receiving the methionine supplement, the average daily growth was 1.7 gm. On the other hand, *l*-cystine had no growth-stimulating effect.

Thus methionine appears to be the limiting nutritive factor in arachin and cannot be replaced by cystine. The results support those of Rose and collaborators⁴ in studies with mixtures of highly purified amino acids. The arachin diet is easily prepared and is useful in the production of a methionine deficiency in rats. It is being employed in further studies of the requirements of animals for various types of organic sulfur.

Further Observations of the Effect of Parenteral Injection of Amino Acids and Related Substances upon Creatine Formation and Storage in the Rat. BY HOWARD H. BEARD, THOMAS S. BOGGESS, AND PHILIP PIZZOLATO. *From the Department of Biochemistry, Louisiana State University Medical Center, New Orleans*

Forty-nine different amino acids and related substances were injected in optimum doses in young adult rats and the increase in muscle creatine above normal observed from 1 to 4 days later. 132 control and about 573 experimental animals were used. Increases up to 138 per cent in muscle creatine were observed. Much evidence for the influence of glycine and the methylation process in the body in creatine formation was obtained. It was suggested that most of the amino acids could be metabolized

³ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

⁴ Rose, W. C., Kemmerer, K. S., Womack, M., Mertz, E. T., Gunther, J. K., McCoy, R. H., and Meyer, C. E., *Proc. Am. Soc. Biol. Chem.*, **8**, lxxxv (1936) (*J. Biol. Chem.*, **114** (1936)).

through glycine in creatine formation. Only glycine and urea, of the intermediate products of amino acid metabolism, increased the muscle creatine above normal.

100 and 200 mg. of urea gave no increase, while 300 mg. gave increases up to 90 per cent above normal. The injection of 100 mg. of urea with 100 mg. of various other substances gave significant increases above that obtained from these substances alone. Conclusive evidence was obtained that urea can be transformed into the guanidine group of creatine with the assistance of the amino acids, the amino group of which is necessary for creatine formation. These observations indicate that the guanidine group of creatine is synthetic in origin, thus explaining both the endogenous and exogenous origin of creatine in the body.

The following theory of creatine formation in the body, with much additional evidence from the literature for its validity, is suggested: amino acids \rightarrow glycine + urea \rightarrow glyocyamine \rightarrow creatine. Increasing the concentration of any of one or more of the components of this reaction results in an increased creatine formation.

The Replaceability of Cystine with Some Partially Oxidized Derivatives. BY MARY A. BENNETT. *From the Lankenau Hospital Research Institute, Philadelphia*

Albino rats were maintained on du Vigneaud's basic cystine-deficient diet.^{*} Different groups were fed, in addition, cystine (Merck), cystine purified by ten recrystallizations, cystine disulfide, and sulfinic acid. Identical growth curves were obtained with cystine (Merck) and the highly purified cystine. Cystine disulfide proved capable of replacing cystine; hence the reaction $\text{—SO—SO—} \rightleftharpoons \text{—S—S—} + 2\text{O}$ is probably reversible in the body. Sulfinic acid produced no growth, the curve closely following that of the controls without cystine, indicating that $\text{—SO}_2\text{H}$ cannot be converted to —SH .

It was desired to use a —SOH derivative, but since sulfenic acid was found too unstable for isolation, thioformamidyl-S-cysteine (synthesized by Dr. Toennies) was fed, with thiourea and dithioformamidine as control. The significance of these compounds as indicated by their ability to replace cystine in the diet is discussed. *

^{*} Dyer, H. M., and du Vigneaud, V., *J. Biol. Chem.*, **115**, 547 (1936).

Hemolytic and Antihemolytic Properties of Bile Acids and Sterols.

BY FRIEDA BERLINES. *From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

The natural bile acids are known to be hemolytic, while cholesterol and other natural sterols inhibit this effect. In view of the similar nuclear structure of these two groups of compounds, the aim of this study has been to associate these effects with certain groupings in the steroid molecule. The natural bile acids and sterols differ in the configuration of the carbinol group at C₃. Epimerization of the carbinol group, whereby the bile acids are converted into compounds having a configuration at C₃ identical with that of the sterols, is always followed by a loss of hemolytic properties. In many cases these epimerized compounds are now actually antihemolytic. With the sterols, epimerization at C₃ leads to a loss of antihemolytic properties. No hemolysis was obtained, however, with these episterols, probably because of the great insolubility of the substances. From this we conclude that the hemolytic and antihemolytic properties of the bile acids and sterols are attendant upon the configuration of the carbinol group at C₃.

Choline Oxidase. BY FREDERICK BERNHEIM AND M. DOROTHY WEBSTER. *From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina*

The alcohol group of choline is rapidly oxidized by liver. The enzyme responsible for the oxidation has been purified and shown to be specific for choline and distinct from the alcohol oxidase. At pH 6.6 choline is quantitatively oxidized to the aldehyde, as shown by the O₂ uptake figures and the formation of a bisulfite compound. At pH 7.9 it is quantitatively oxidized to the acid. The oxidation is inhibited by relatively large amounts of cyanide, urethane, physostigmine, and fluoride. Methylene blue is reduced.

The Amino Acid Composition of Neuroproteins and the Protein of the Neurofibrils. BY RICHARD J. BLOCK. *From the Department of Chemistry, New York State Psychiatric Institute and Hospital, New York*

The observation by Ewald and Kühne (1877) that lipid-extracted nerve tissues are extraordinarily resistant to enzymatic digestion has been amply confirmed (Chittenden, Argiris, Nelson, Block). Furthermore, I have shown that neurokeratin is *not* a true keratin in the sense of Block and Vickery who have classified keratins on the basis of amino acid composition.

Experiments still in progress appear to indicate that *neurokeratin is one of the principal proteins of the brain* and that a collagen is the chief contaminating protein in dried defatted nervous tissue. The evidence on which this statement is based is the following: (1) Total brain protein refluxed with water yielded approximately 40 per cent of its weight, as a water-soluble protein, presumably gelatin. (2) Neurokeratin, collagen, and total brain protein, respectively, gave the following analytical figures: arginine 4.0, 9.0, 5.3; lysine 3.1, 6.0, 4.0; histidine 1.8, 0.9, 1.4; tyrosine 7.0, 0.0, 3.8; cystine 3.7, 0.0, 3.8; tryptophane 1.5, 0.0, 0.9 per cent. (3) The amino acid composition of total brain protein calculated as made up of 55 per cent neurokeratin and 45 per cent collagen (gelatin) agrees with the values actually found.

Nerve cells and their axones contain discrete, continuous filaments often of remarkable length, the neurofibrils, which are probably of protein nature and which are extraordinarily resistant to autolytic and degenerative decomposition. The insolubility of neurokeratin and its resistance to enzymatic digestion (properties common to many long fibrous proteins and probably enhanced by treatment with lipid solvents) indicate that it may be the protein of the neurofibrils (neurofibrillin).

The Metabolism of *dl*- γ -Methiol- α -Hydroxybutyric Acid in Cystinuria. BY RICHARD J. BLOCK, ERWIN BRAND, AND GEORGE F. CAHILL. *From the Department of Chemistry, New York State Psychiatric Institute and Hospital, and from the Squier Urological Clinic of the Presbyterian Hospital, New York*

The fate of *dl*- γ -methiol- α -hydroxybutyric acid ($\text{CH}_3\text{SCH}_2\text{CH}_2\text{CHOHCOOH}$) (I) was investigated in a case of cystinuria (patient A). This compound, like cysteine, homocysteine, and methionine, yields extra cystine in the urine. Following the ingestion of 8 gm. of (I) the extra cystine amounted to 30 per cent and inorganic sulfate to 25 per cent of the extra S. Undetermined neutral S

accounted for 43 per cent of the extra S in this experiment, while in feeding experiments with *DL*-methionine and *DL*-homocysteine this sulfur fraction was only 15 and 25 per cent respectively. This discrepancy in the undetermined neutral S remains unexplained, but further light may be shed on this question by feeding experiments with the optical isomers of (I).

We are inclined to interpret the above experimental results to indicate that the extra cystine excreted in the urine was derived from (I). It appears, therefore, that the intermediary metabolism of a non-nitrogenous compound may result in the excretion of an amino acid. These experiments furnish additional evidence that one of the pathways of methionine catabolism is its conversion into cysteine and the results are compatible with the working hypothesis recently suggested⁶ for this conversion.

Excretion of Ingested Guanidoacetic Acid. BY METER BODANSKY, VIRGINIA B. DUFF, AND CORNELIUS L. HERRMANN. *From the Laboratories of the John Sealy Hospital and the Department of Pathological Chemistry, University of Texas, Galveston*^{*}

The adult rat normally excretes approximately 1 mg. of guanidoacetic acid per day. On feeding 100 mg. of this substance by stomach tube, an extra excretion of 40 to 75 mg. resulted. The same amount given in four equally divided doses did not affect this high level of excretion significantly. When the quantity was reduced to 40 mg. given in four 10 mg. doses, the recovery averaged 22 mg., or 55 per cent. These observations are based on the results of long continued (48 to 53 days) and carefully controlled experiments on each of six male and six female rats, weighing 250 to 300 gm. The urines were collected daily and analyzed for total nitrogen, creatine, creatinine, and guanidoacetic acid.

In human subjects (two male, one female), 2 gm. of guanidoacetic acid taken by mouth produced an average increase of 470 mg. (23 per cent excretion). The same amount taken in six divided doses at 2 hour intervals augmented the urinary output by an average of 395 mg. (20 per cent excretion). The extra excretion resulting from a 1 gm. dose varied from 172 to 207 mg. (average 186 mg.). Retention was definitely better when the

^{*} Brand, E., Block, R. J., Kassell, B., and Cahill, G. F., *Proc. Soc. Exp. Biol. and Med.*, **35**, 501 (1936).

same amount was taken in six divided doses at 2 hour intervals. Only 79 to 112 mg. (average 105 mg.) of extra guanidoacetic acid were present in the urine.

From these results it would seem that the tolerance for guanidoacetic acid is easily overstepped. Provided this compound is in reality an important intermediate in metabolism, it may be surmised that under normal conditions its production in the tissues occurs at a rate sufficiently slow to assure almost complete utilization.

Some Observations on the Source of Urinary Cystine in Cystinuria.

BY ERWIN BRAND, RICHARD J. BLOCK, AND GEORGE F. CAHILL.

From the Department of Chemistry, New York State Psychiatric Institute and Hospital, and from the Squier Urological Clinic of the Presbyterian Hospital, New York

The metabolism of casein and of lactalbumin was investigated in a case of cystinuria. The methionine to cystine ratio of casein was 9 and that of lactalbumin 1. Under the conditions of the experiments, methionine and cystine fed as constituents of casein and lactalbumin were catabolized both qualitatively and quantitatively in the same way as when they were administered in the form of the free amino acids. Methionine and cystine were catabolized in the same ratios in which they are present in casein and in lactalbumin.

Detailed analysis of the data indicates that, at the present state of our knowledge of the composition of casein and of lactalbumin, a true picture of the catabolism of these proteins and of the composition of "storage" protein cannot be obtained from an examination of the N and S output alone or in combination. The results are in agreement with previous suggestions that one of the pathways of methionine catabolism is its conversion into cysteine and that the cystine excreted in cystinuria is derived from dietary methionine.

The catabolism of carboxymethyl-S-cysteine (I) in both normals and cystinurics leads to the excretion of a mixed disulfide, $\text{HOOC} \cdot \text{CH}_2 \cdot \text{S} \cdot \text{SCH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$ (II). (I) is apparently the source of the thioglycolic acid moiety of (II), but the cysteine moiety of (II) may be furnished either by (I) or by cysteine available

in the body. The working hypothesis¹ recently suggested² for the conversion of methionine into cysteine was based in part on these results.

Excretion of Fixed Base during the Alkalosis of Overventilation.

By A. P. BRIGGS. *From the Departments of Biochemistry and Internal Medicine, University of Georgia School of Medicine, Augusta*

An attempt has been made to determine the factors responsible for the alkaline urine secreted during the alkalosis of overventilation. Acids and bases have been determined in the urine before, during, and after periods of overventilation. The results indicate that the alkaline urine is due in part to the accompanying diuresis and diminished excretion of chloride. A large portion of the extra fixed base excretion remains unaccounted for, and the results indicate that this is not due to decreased production of ammonia.

The Metabolism of *d*- and *dl*-Glutamic Acids, *l*- and *dl*-Aspartic Acids, *dl*-Pyroglutamic Acid. By JOSEPH S. BUTTS, MAX S. DUNN, AND HARRY BLUNDEN. *From the Department of Biochemistry, University of Southern California School of Medicine, and the Chemical Laboratory, University of California at Los Angeles, Los Angeles*

Glycogen was determined on the livers of male rats previously fasted 48 hours at 2, 4, 6, 8, 10, and 12 hour intervals after the feeding of the various amino acids. Diarrhea was almost completely avoided by giving filter paper to the animals during the preliminary fast period. A bacteriological study failed to show cellulose-splitting organisms present in the gastrointestinal flora and the control animals showed no more glycogen than a group similarly treated but receiving no cellulose; this procedure thus seemed justified.

The maximum absorption of the amino acids which were fed as the monosodium salts (except pyroglutamic which was given as the free acid) was obtained by administering hourly by stomach tube an amount slightly in excess of that capable of being absorbed.

¹ Brand, E., Bloch, R. J., Kassell, B., and Cahill, G. F., *Proc. Soc. Exp. Biol. and Med.*, **35**, 501 (1936).

The results uniformly showed glycogen deposition, particularly in the longer periods, with *L*-aspartic, the most effective, followed in turn by *DL*-aspartic acid, *DL*-pyroglutamic acid, *D*-glutamic acid, and *DL*-glutamic acid. The values seem to substantiate a finding reported from this laboratory in a study of *D*- and *DL*-alanine; namely, that the natural isomer is more effective as a glycogenic agent than is the racemic mixture.

As confirmatory evidence of their glycogenic ability, these same compounds have been fed to animals receiving sodium butyrate. A decrease in acetone body excretion after administration of the amino acid is considered indicative of ketolytic activity. The study showed *L*-aspartic acid the best in this property, followed in turn by *DL*-pyroglutamic acid, *D*-glutamic acid, and *DL*-aspartic acid, when isomolecular amounts were given.

Male Hormone in the Urine of Bulls and Rams. BY LEWIS W.

BUTZ AND S. R. HALL. *From the Bureau of Animal Industry and the Bureau of Dairy Industry, National Agricultural Research Center, United States Department of Agriculture, Beltsville*

Although crystalline compounds with male hormone activity have been isolated from bovine testicles (testosterone) and adrenals (adrenosterone), no active excretion products from this species have been reported. On the other hand, male human urine has yielded two active substances (androsterone and dehydroandrosterone) and active fractions have also been obtained from the urine of women.

We have now found that bulls and rams excrete active substances in the urine. The procedure used for extracting the active fraction combined the essential features of those of Gallagher, Koch, and Dorfman⁸ and Callow.⁹ The urines were first brought to approximately pH 1.0. Much more acid was required to accomplish this with our bull and ram urines than with our male human urines. 20 cc. of concentrated HCl per liter of urine in addition were then added, and the urine filtered and extracted with benzene. The phenol fraction was very large and was removed before assaying by washing with 4 per cent NaOH.

⁸ Gallagher, T. F., Koch, F. C., and Dorfman, R. I., *Proc. Soc. Exp. Biol. and Med.*, **33**, 440 (1935).

⁹ Callow, R. H., *Lancet*, **2**, 565 (1936).

Tricaproin or olive oil was used as solvent for injecting rats and capons with testosterone and the urine fractions. Tricaproin is the preferred solvent.

The Separation of Glycogen into Two Fractions. BY DAN H. CAMPBELL. *From the Department of Hygiene and Bacteriology of the University of Chicago, Chicago*

It has been found that ordinary preparations of glycogen can be separated by careful fractional precipitation, with either ethyl alcohol or ammonium sulfate, into two chemically different fractions designated here as α - and β -glycogen.

Preliminary investigation was made of glycogen from mussels and rabbit liver. Three methods were used for extraction: first, with hot NaOH; second, with hot distilled water; and third, with cold distilled water plus a small amount of lead acetate. The α : β ratios obtained by the first two methods were 3.2 to 3.5 for rabbit liver and 2.5 to 3.0 for mussels. The ratio was much lower when tissue was extracted in cold-distilled water. There was little change in the above ratios when glycolysis was allowed to take place at 37° for a short time.

The α fractions, regardless of method used for extraction, produced very opalescent solutions in 0.5 per cent concentration, and contained a trace of nitrogen and phosphorus and no ash. The $[\alpha]_D^{24}$ ranged from +250° to +320°. The β fractions produced only faintly opalescent solutions in 1.0 per cent concentration, contained a trace of nitrogen, a much greater amount of phosphorus than the α fractions, and gave 0.1 to 0.5 per cent ash. The $[\alpha]_D^{24}$ for β fractions was +150° to +160° for 1 per cent solutions. The rate of hydrolysis, as indicated by copper reduction, was approximately the same for both fractions. The final reducing value for all fractions was approximately 100 per cent, calculated as glucose.

The Use of Nembutal in the Determination of the Basal Metabolic Rate of Dogs. BY J. W. CAVETT. *From the Laboratory of Physiological Chemistry, University of Minnesota, Minneapolis*

The determinations were made with a Benedict-Roth metabolism machine. The mouthpiece was made of a can into the bottom of which a tube was soldered for attaching to the machine. The

open end of the can was closed by a rubber dam having a small hole in its center. This enclosed the dog's nose and mouth so that there was no leakage of gases.

Four dogs which were trained to lie quietly for metabolic studies were used. The determinations were made after the dog had lain quietly for half an hour. As soon as this determination was completed, the dog was given 25 mg. of nembutal per kilo of body weight intraperitoneally in 5 cc. of water. The dog fell asleep within 12 to 15 minutes and could not be aroused for at least 1½ hours. The rectal temperature was taken before giving the nembutal and maintained by keeping the dog covered.

Four to seven sets of determinations were made upon each dog. 1 hour after receiving the nembutal the metabolic rates were the same as those obtained with the dog in a basal state without nembutal.

48 hours after having received 10 mg. of thyroxine intravenously, a dog, which on previous determinations had a basal metabolic rate of 27 calories per hour, gave rates of 37.6 calories without and 30.4 with nembutal. However, the dog was very irritable without the nembutal and was not in a true basal condition.

Blood Plasma Proteins in Partially Nephrectomized Rats. By

ALFRED CHANUTIN AND STEPHAN LUDEWIG. *From the Biochemical Laboratory, University of Virginia, University*

Hypoproteinemia may follow excessive loss of protein or protein deprivation. Bloomfield has suggested that an impairment of blood protein regeneration and not "loss or lack" of protein is primarily responsible for the hypoproteinemia. In the present investigation it has been demonstrated that the continued proteinuria in partially nephrectomized rats does not appreciably affect the concentration of the plasma proteins. These animals were fed diets containing widely varying concentrations of protein. This study substantiates Bloomfield's hypothesis that loss of protein *per se* is not sufficient explanation to account for hypoproteinemia.

The Utilization of Carotene by the Human Fetus. By S. W.

CLAUSEN AND A. B. MCCOORD. *From the Department of Pediatrics, The University of Rochester School of Medicine and Dentistry, Rochester, New York*

Since the carotenoid pigments are present in very low concentration in the cord blood of the human fetus, it seemed important to determine, if possible, by *in vitro* experiments, whether the polyenes are rapidly and wastefully destroyed by the fetus. Carotene was the pigment studied first. To determine the effect of pH, stable colloidal suspensions were prepared by adding 0.1 N HCl, 0.01 N HCl, and phosphate buffer solutions of pH 6.0, 7.0, and 8.0, each containing 1 per cent gelatin, to the pigment dissolved in a small amount of acetone. When these suspensions were incubated at 37° and aerated for approximately 6 hours, very little destruction occurred, indicating that carotene is not very sensitive to oxidation at these concentrations of H ion. The apparatus for the aeration was so arranged that, after the acetone had evaporated, no further change in volume took place.

Suspensions of carotene in normal defibrinated adult blood and serum were treated in the same manner and no oxidation of carotene could be detected. Similar results were obtained with defibrinated cord blood and serum of the human fetus, so that thus far no evidence has been acquired that carotene is rapidly destroyed by any constituent in the blood of the fetus. Mixtures of fetal and adult blood with acetone-free colloidal suspensions of carotene yielded the same results.

Carotene was fairly rapidly destroyed under the above conditions when suspended in the Ringer's solution supplied by the University Hospital, but this solution may contain metallic ions which catalyze the reaction. Carotene suspended in 0.85 per cent NaCl was not oxidized.

The Isolation and Synthesis of Glucose-1-Phosphoric Acid. BY CARL F. CORI, GERTY T. CORI, AND S. P. COLOWICK. *From the Department of Pharmacology, Washington University School of Medicine, St. Louis*

When glycogen, inorganic phosphate, and adenylic acid (2 mg. per 100 cc.) are added to a dialyzed rabbit muscle extract, α -glucose-1-phosphoric acid is formed as the first phosphorylation product, which was isolated as the barium salt. This ester is converted to hexose-6-phosphoric acid by an enzyme present in the dialyzed extract, a reaction which is greatly accelerated by the addition of magnesium ions.

The ester was synthesized by refluxing for 1 hour α -tetraacetyl-

1-bromoglucose with the theoretical amount of freshly precipitated silver phosphate in dry benzene. An intermediate containing 2.76 per cent P was isolated in crystalline form; theoretical for tri-(tetraacetylglucose-1)-phosphoric acid, molecular weight 1088, 2.84 per cent P, $[\alpha]_D^{25} = +122^\circ$ (in methanol). The intermediate was dissolved in 0.2 N HCl in methanol and kept overnight at 25° . The solution was made alkaline with barium hydroxide and the precipitate centrifuged off, which contained the barium salt of glucose-1-phosphoric acid.

The identity of the natural and synthetic products was established by: (1) the specific rotation $[\alpha]_D^{25} = +75^\circ$ (for the dry barium salt in water), $+120^\circ$ (for the free acid); (2) the velocity constant during hydrolysis in 0.25 N HCl at 37° , $K = 1.30 \times 10^{-3}$ for both the glucose and phosphoric acid split off; (3) the behavior, when added to muscle extract. In a dialyzed extract both esters yield hexose-6-phosphoric acid (Embden ester) which was isolated as the barium salt and identified. Addition of magnesium ions accelerates this conversion. In a non-dialyzed extract or in a shortly dialyzed extract reactivated by adenosinetriphosphate and magnesium both esters yield lactic acid.

Catabolism of Amino Acids with Branched Chains. BY RALPH C. CORLEY AND FRED H. SNYDER. *From the Laboratory of Biochemistry, Department of Chemistry, Purdue University, Lafayette*

Amino acids with branched chains have been administered parenterally to dogs maintained in nitrogen balance. The urine has been analyzed for total nitrogen, urea nitrogen, and amino acid nitrogen. The nitrogen of 2-amino-2-methylpropanoic acid, 2-amino-2-methylbutanoic acid, *d*-(-)-valine, and *d*-(-)-isoleucine, after administration, was largely accounted for by increases in amino acid nitrogen, with no significant increases in urea nitrogen. The administration of *l*-(+)-valine, *l*-(+)-isoleucine, *l*-(-)-leucine, and *d*-(+)-leucine, respectively, was followed by no significant increases in amino acid nitrogen, but by increases in urea nitrogen in amounts to account for that of the compounds injected.

The following conclusions have been drawn for the amino acids studied in the experimental conditions employed. A methyl group attached to the carbon atom carrying the amino group

interferes with deamination. A methyl group attached to the carbon atom, the second from that carrying the amino group, has been found not to interfere with deamination. A methyl group attached to the carbon atom next to that carrying the amino group does not interfere with deamination if the amino acid has the natural (*l*) form, but does interfere with deamination of the amino acid with the antipodal form.

To extend this series of observations we are synthesizing pseudoleucine (2-amino-3-trimethylpropanoic acid) and *d*- and *l*-alloisoleucine (epimers of *d*- and *l*-isoleucine).

Decreased Sensitivity to Theelol of Castrate Female Rats. BY JACK M. CURTIS, LLOYD C. MILLER, AND EWALD WITT. *From the Laboratories of the Division of Pharmacology, Food and Drug Administration, United States Department of Agriculture, Washington*

The estrogenic potency of theelol has been reported by Curtis and Doisy¹⁰ as 0.65 microgram per rat unit, and by Meyer, Miller, and Cartland¹¹ as 200 micrograms per rat unit. Notwithstanding this enormous assay difference, the physical data on these two samples have led to the belief that they are structurally identical. Samples of theelol from both laboratories have been obtained by us, and a comparison of their estrogenic potencies has established their biological identity.

Of more fundamental importance, however, is the observation of the reactions of castrate female rats used repeatedly for the assay of theelol. Our rats respond to an initial injection of theelol at a dose level comparable to that reported by Curtis and Doisy. Subsequent injections of larger doses are ineffective, and the threshold gradually increases to a value some 50 or more times the initial dose. During this period of time the response of these animals to international standard theelin does not change. Thus the rats lose sensitivity to theelol but show no change in sensitivity to theelin. Present indications are that this loss of sensitivity is related neither to the age of the animal nor to the interval between castration and the first administration of theelol.

¹⁰ Curtis, J. M., and Doisy, E. A., *J. Biol. Chem.*, **91**, 647 (1931).

¹¹ Meyer, R. K., Miller, L. C., and Cartland, G. F., *J. Biol. Chem.*, **112**, 597 (1935-36).

The Influence of Different Casein Preparations in Flavin-Deficient Diets upon the Appearance of Cataract. BY PAUL L. DAY AND WILLIAM J. DARBY. *From the Department of Physiological Chemistry, School of Medicine, University of Arkansas, Little Rock*

In the investigation of the influence of various conditions upon the appearance of cataract in rats from flavin deficiency, five casein preparations were used. The diets were identical except for the caseins. One sample was a crude casein which we washed with 60 per cent alcohol. The other four samples were commercial "vitamin-free" preparations. Cataract appeared regularly in rats receiving the alcohol-extracted and two of the "vitamin-free" products. With these two caseins growth ceased in nearly all of the animals before the end of the 4th week. The alcohol-extracted casein promoted slightly greater and longer growth, and the appearance of cataract was delayed somewhat. The other two commercial "vitamin-free" caseins were purchased from one manufacturer, and are preparations that have been widely used in studies on the B vitamins. Rats receiving these caseins continued growing for 8 weeks or longer, and the average net growth in 10 weeks was about 6 times greater than that in animals receiving caseins that were strictly flavin-free. Less than 20 per cent of the rats receiving these two caseins developed cataract in the first 10 weeks of experiment. Eluates from these two caseins exhibited a greenish fluorescence, characteristic of lactoflavin, when viewed under ultraviolet light.

It appears probable that the failure of some investigators to observe cataract in rats as a result of flavin deficiency may have been due to the contamination of their caseins with lactoflavin.

The Relation of Ketonuria to Liver Fat. BY HARRY J. DEUEL, JR., LOIS F. HALLMAN, AND SHEILA W. MURRAY. *From the Department of Biochemistry, University of Southern California School of Medicine, Los Angeles*

The ketonuria of rats during a 5 day fast following a 14 day period in which a high fat diet was fed was greater in female than in male animals. The height of the ketonuria was independent of the level of liver fat. Thus, there was as great a ketonuria

after giving cod liver oil as after feeding a diet containing butter fat, coconut oil, or lard and cholesterol. Liver fat was lowest in the cod liver oil tests (8 to 13 per cent); higher after coconut oil (28 per cent); and highest with butter fat (32 to 35 per cent) and lard-cholesterol (35 to 38 per cent). Practically no ketonuria developed following our stock diet, and liver fat was at a normal level (3 to 4 per cent). Maximum ketonuria was obtained on the 2nd or 3rd day, followed by rapid fall. Liver fats were usually slightly higher in female than in male rats at the start of the fast; however, following a 3 or 5 day fast the average decrease in liver fat in the females far exceeded that in the males in all cases. It is postulated that the greater ketonuria in the female may be traceable to the deposition of a more labile fat which disappears from the liver more rapidly on fasting. The rate of depletion of fat from the liver was slowest in those rats which had received cholesterol in the diet. A sex difference in glycogen was always noted in the unfasted animals. The glycogen store in female rats was 47 to 73 per cent of that in the males.

The Physicochemical Properties of Human Blood at High Altitudes. BY D. B. DILL, JOHN H. TALBOTT, AND W. V. CONSOLAZIO. *From the Fatigue Laboratory, Morgan Hall, Harvard University, and the Medical Clinic of the Massachusetts General Hospital, Boston*

In the arterial blood of resting man partially or fully acclimatized to altitudes as high as 6.14 km. the most notable change in electrolytes is the decrease in combined CO_2 . At 5.34 km. serum bicarbonate is about 8 milli-equivalents less than at sea-level. The distribution between serum and cells remains unchanged.

The decrease in CO_2 is partially balanced by an increase of 4 milli-equivalents in serum chloride and a decrease of about 2 milli-equivalents in sodium, leaving an unexplained anion deficit of about 2 milli-equivalents. Lactate, proteinate, and osmotic pressure remain constant. The pH increases slightly.

In comparison with man at sea-level, the fully acclimatized man at 5.34 km. has about one-half more hemoglobin and red cells per unit volume of blood. The buffer value of his oxygenated blood is about one-third greater. His reduced alkaline reserve and

$p\text{CO}_2$ make his capacity to neutralize fixed acid at constant pH less than that of man at sea-level.

A comparison of man at sea-level (first figure) with man fully acclimatized to 5.34 km. (second figure) is as follows: total Hb 9.0, 13.5 mm; HbO_2 8.6, 10.3 mm; arterial $p\text{O}_2$ 42, 88 mm. of Hg; venous $p\text{O}_2$ 30, 37 mm. of Hg; venous $p\text{O}_2$, moderate work, 21, 21 mm. of Hg.

The Comparative Activity of Various Estrogens on the Infantile Rat Uterus and Vagina. BY R. I. DORFMAN. *From the Department of Pharmacology and Experimental Therapeutics, Louisiana State University Medical Center, New Orleans, and the Laboratory of Physiological Chemistry and the Adolescence Study Unit, Yale University School of Medicine, New Haven*

The quantitative response of the infantile rat uterus and vagina to theelol, theelin, theelin benzoate, dihydrotheelin, dihydrotheelin benzoate, equilin, equilenin, testosterone, androstenediol, and androstenediol benzoate was investigated. Olive oil solutions of the estrogens were administered subcutaneously daily for 5 days into 25 day-old female rats. 24 hours after the last injection the animals were sacrificed. Body weight, uterine weight, and vaginal introitus were determined.

In amounts up to 200 micrograms per day androstenediol benzoate was found to be inactive. Testosterone and androstenediol in amounts of 200 to 400 micrograms per day were effective in causing uterine and vaginal stimulation. The other estrogens investigated were active on the uterus and vagina in amounts ranging from 0.06 to 1.00 microgram per day, depending on the substance administered. In the following two lists these substances are arranged in the order of their activity as gaged respectively by uterine and vaginal response. In both cases the most active material is listed first: (1) dihydrotheelin, dihydrotheelin benzoate, theelin, equilin, equilenin, theelin benzoate, and theelol; (2) dihydrotheelin, dihydrotheelin benzoate, theelin, equilin, theelol, equilenin, and theelin benzoate. It is of interest to note the remarkable decrease of activity of theelin on the uterus and vagina when it is converted to its benzoate, while no significant difference of activity was noted between dihydrotheelin and its monobenzoate.

The Excretion and Metabolism of Theelin in the Sexually Immature Rat. BY R. I. DORFMAN. *From the Department of Pharmacology and Experimental Therapeutics, Louisiana State University Medical Center, New Orleans, and the Laboratory of Physiological Chemistry and the Adolescence Study Unit, Yale University School of Medicine, New Haven*

Balance studies were made on the excretion and metabolism of theelin in the sexually immature male and female rat. 28 day-old rats of both sexes were injected subcutaneously with 25 micrograms of theelin dissolved in olive oil. Control animals received injections of olive oil alone. The urine and feces were collected quantitatively for 5 days and the animals were then sacrificed. The urine plus the feces, the carcasses, and the food were hydrolyzed separately with hydrochloric acid and extracted with benzene in a continuous extractor. The estrogenic activity of the extracts was assayed on the spayed adult rat.

The control male and female rats excreted less than 1 microgram of theelin each in the urine and feces during the 5 day test period. The average theelin content of the carcasses of these animals was less than 2 micrograms. The theelin-injected males and females excreted 2.5 micrograms of theelin per rat or 10 per cent of the amount injected, and the carcasses of these animals contained less than 7 micrograms of theelin each. An analysis of the food showed that the animals received less than 0.5 microgram of theelin from that source during the 5 day period of the experiment. Of the 25 micrograms of theelin injected, at most only 9.5 micrograms could be accounted for in the excretions and in the carcasses. The remaining 15.5 micrograms, or approximately 60 per cent, must, therefore, have been destroyed or inactivated in the body of the immature animal during the 5 day test period. No sexual difference in the excretion and metabolism of theelin was noted in the animals studied.

The Response of the Chick Comb to Sex Hormones. BY R. I. DORFMAN AND WILLIAM WALTER GREULICH. *From the Laboratory of Physiological Chemistry and the Department of Anatomy and the Adolescence Study Unit, Yale University School of Medicine, New Haven*

The response of the white Leghorn chick comb to andro-

sterone, testosterone, transdehydroandrosterone, theelin, and dihydrotheelin was investigated. Olive oil solutions of these substances were injected subcutaneously into 7 day-old chicks for 6 consecutive days. The animals were killed 48 hours after the last injection and their combs were carefully removed and weighed.

The comb response of male and female chicks to the injection of various amounts of androsterone was determined. It was found that testosterone and transdehydroandrosterone also elicited a comb response from chicks of both sexes. Neither theelin nor dihydrotheelin was effective in accelerating comb growth, even when administered in amounts of 25 micrograms per day for 6 days.

Approximately 1000 carefully selected chicks were used in this study.

A New Graphic-Mathematical Analysis of Absorption Spectra, As Applied to Hemoglobin Derivatives. BY DAVID L. DRABKIN. *From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

The location of the maxima in the absorption spectra (visible and ultraviolet) of various hemoglobin derivatives can be predicted by the equation, $n = (\nu \times 10^{-2}) / (\nu_0 \times 10^{-2})$, where n is an integer (4, 5, 6, 7, 8, 9, 10, and 11) and $\nu_0 \times 10^{-2}$, the fundamental wave number, is 40. Two other maxima, α and β , not in the above series, are also present in the spectra of all the derivatives studied. These two maxima in the visible green region are obvious in the spectra of gaseous derivatives of hemoglobin, alkaline methemoglobin, and pyridine hemochromogen. Analysis yielded the interesting result that the single visible band in the spectrum of cyanmethemoglobin is composed of four components, the No. 4 and 5 bands of the above series and the α and β bands, the latter occupying the customary position of the corresponding bands in the hemochromogen spectrum.

The complex absorption curves represent the summation of individual bands whose shapes may be described by normal frequency curves of the form, $y = ke^{-\frac{(x-a)^2}{2\sigma^2}}$. The summation,

Σ , of unit curves of this type reproduces accurately the total absorption curve, which is mathematically given by $\Sigma(y_a = ke^{\frac{(x-(n \cdot 40))^2}{2\sigma^2}}) + \Sigma(y_a, y_\beta)$.

The analysis has led to the following tentative suggestions. (a) Globin, contrary to prevalent concept, is not largely responsible for the absorption in the ultraviolet region. It probably serves as a "resonator," influencing the intensity but not the pattern of the bands. (b) Ferro and ferri derivatives of hemoglobin have similar patterns. (c) The intensities of the α and β bands are probably partly a function of the degree of dissociation of the derivatives studied.

The Nature of the Estrus-Inhibiting Substances in Testis Tissue Concentrates. BY D. R. L. DUNCAN, T. F. GALLAGHER, AND F. C. KOCH. *From the Department of Biochemistry of the University of Chicago, Chicago*

Testis tissue concentrates which still contain phospholipids and sterols when injected into adult rats in doses of 1 international androgenic unit inhibit the estrous cycle. Further fractionations in parallel with assays for androgenic activity on capons and estrus-inhibiting activity on normal adult rats in doses equivalent to the original androgenic unit show that the estrus-inhibiting action does not fall into the androgenic fraction. Lecithin and cephalin of high degrees of purity, prepared from the original tissue concentrate, possess the estrus-inhibiting potency in sufficient degree to account for most of the estrus-inhibiting activity in the original concentrate. The hydrolyzed phospholipids and synthetic choline and cholamine possess the estrus-inhibiting action of approximately the same order on M equivalent doses. Crystalline testosterone in doses of 20 international androgenic units (0.30 mg.) does not inhibit the estrous cycle. Obviously the estrus-inhibiting activity does not parallel the androgenic activity in impure testis tissue concentrates. Estrus inhibition is not a reliable biological test for male hormone assay. The physiological mechanism involved in the inhibition has not been determined.

The Solubility of Certain Amino Acids in Water-Ethyl Alcohol Mixtures. BY MAX S. DUNN, FRANK J. ROSS, AND M. PALMER STODDARD. *From the Chemical Laboratory, University of California at Los Angeles, Los Angeles*

In these studies the purity of the amino acids was established by glass electrode titrations. All analyses, except those of *d*-glutamic acid and *dl*-aspartic acid, were carried out in the presence of formaldehyde. The solubilities of *d*-glutamic acid, *dl*-valine, *dl*-aspartic acid, glycine, *dl*-alanine, *dl*-norleucine, *dl*-leucine, and *dl*-serine in 25, 50, 75, and 95 per cent ethyl alcohol at 0°, 25°, 45°, and 65° were determined by the gravimetric method described in an earlier paper.

The authors' experimental solubility values have been compared with the data in the literature and they have been correlated with alcohol concentration, temperature, dielectric constant of the solution, and other factors which characterize the behavior of amino acids in solution.

They have been further utilized in calculating solubility ratios which denote the relative weights of amino acids that remain dissolved and precipitate on recrystallization. The numbers derived by simple calculation from the solubility ratios are named *crystallization constants*. Constants may be determined for water alone, for any desired concentrations of ethyl alcohol in water, or for any ethyl alcohol solution and water; those calculated on the latter basis are the most useful, since amino acids are commonly recrystallized by adding ethyl alcohol to their aqueous solutions. Hence, crystallization constants have been calculated from the expression

$$K = \frac{\text{Gm. amino acid per 100 gm. aqueous alcohol at } 0^{\circ} \times \text{dilution factor}}{\text{Gm. amino acid per 100 gm. water at } 100^{\circ}}$$

When these values are plotted against per cent ethyl alcohol and curves drawn through the points, the resulting graphs may be used to determine the optimum conditions under which the amino acids under investigation may be recrystallized.

The Metabolic Behavior of Djenkolic and Homodjenkolic Acids.

BY HELEN M. DYER. *From the Department of Biochemistry, School of Medicine, George Washington University, Washington*

The recent demonstration that djenkolic acid is the cysteine thioacetal of formaldehyde raised the question whether this compound could yield cysteine in the animal body. The occurrence of the compound in the djenkol bean and the possibility of its occurrence in other legumes emphasized the desirability of carrying out an investigation of this question. It was found that djenkolic acid could not support the growth of white rats maintained on a cystine-deficient diet.

However, it occurred to us that the next higher symmetrical homologue of djenkolic acid (*homodjenkolic acid*) might be utilized for growth under these conditions, since it may be regarded as a derivative of methionine, while djenkolic acid itself is in a certain sense a derivative of S-methylcysteine. This possibility was suggested by consideration of the difference in the metabolic behavior of S-methyl derivatives of cysteine and homocysteine respectively, since it has been previously shown that S-methylcysteine fails to support the growth of animals on a cystine-deficient diet, whereas S-methylhomocysteine (methionine) readily supports growth under these conditions.

The homodjenkolic acid was synthesized by reactions similar to those used in the synthesis of djenkolic acid. *dl*-Benzylhomocysteine was reduced in liquid ammonia and treated with methylene chloride and the inactive homodjenkolic acid was isolated from the reaction mixture. The growth experiments with the homodjenkolic acid demonstrated, however, that it likewise was not available.

The Bromometric Determination of Phenols and Related Substances. BY BEATRICE G. EDWARDS. *From the Department of Biochemistry, University of Oklahoma Medical School, Oklahoma City*

The quantitative behavior of 300 phenols and related substances with 0.1 N bromate-bromide reagent, at 25° in 2.4 N hydrochloric acid, has been investigated both by direct titration, with methyl orange as indicator, and by a modified Koppeschaar method, with excess bromate (5 cc. of 0.1 N bromate per cc. of dilute phenol solution) reacting 30 minutes in the dark.

As might be expected, the majority of substances used more bromine in Koppeschaar's method, where an excess is present.

However, a number of substances, contrary to general experience with ordinary phenols, showed greater bromine uptake by direct titration. These were chiefly *m*-substituted phenols and arylamines, such as resorcinol, *m*-phenylenediamine, *m*-aminophenol, phloroglucinol, and β -naphthol. A very few *o*- and *p*-substituted phenols (quinhydrone and guaiacol), indole, glutathione, and uracil reacted similarly. Since a fraction of the bromine taken up by these substances is in a form which reacts with hydriodic acid, but not with methyl orange, it is evident that the use of potassium iodide in Koppeschaar's procedure complicates the bromometric determination of phenols. This reactive form of bromine is present even before the end-point is reached in the direct titration.

The quantitative data show that *m* substituents in these phenols conform in their effects to well known orientation rules, carboxyl and keto groups being least and hydroxyl and amino groups most effective in producing this phenomenon. The same influences which direct bromine entry into aromatic molecules also lead to the formation of the reactive form of bromine.

The Exchange of Salt and Water between Muscle and Blood in Experimental Chronic Hydronephrosis. BY LILLIAN EICHELBERGER. *From the Lasker Foundation for Medical Research and the Department of Medicine of the University of Chicago, Chicago*

The exchange of salt and water between muscle and blood in experimental hydronephrosis in dogs was studied by contrasting the shifts in water and electrolytes in hydronephrosis with the shifts found in normal dogs under the same experimental conditions.

The experimental hydronephrosis was produced in the animals in two stages: first, the ureter of one kidney was partially constricted about 1.5 inches below the pelvis; and second, within 14 days a nephrectomy on the opposite side was performed. Periods varying from 3 to 6 months were required to produce the terminal uremic stages of hydronephrosis.

Experiments on dogs in terminal stages of hydronephrosis are described in which the extra- and intracellular phases of muscle were studied after an increase in the total body water was produced by the rapid intravenous injection of large volumes of moderately

alkaline isotonic salt solutions. From these data, it was concluded that in the terminal stages of hydronephrosis the original kilo of muscle and the extracellular phase of the muscle increased more in volume than did the muscle in normal dogs under the same experimental conditions.

Comparative Biological Value of Vitamin A As an Alcohol and Ester. BY A. D. EMMETT AND O. D. BIRD. *From the Research Laboratories, Parke, Davis and Company, Detroit*

In our laboratories we have found with vitamin A determinations on fish liver oils by the U.S.P. biological and the spectrographic procedures that the factor for converting $E_1^{1\%}$ cm. at 328 into U.S.P. units is close to 2000. In the case of concentrates prepared from oils high in vitamin A, however, the conversion factor seems to vary with the amount of caustic employed in the saponification.

Three concentrates were prepared: I, partially saponified, where a small amount of oil was left unattacked; II, strongly saponified, where the theoretical amount of alkali was used; and III, completely saponified. In making the biological and spectrographic assays of these concentrates the respective conversion factors were found to be 1970, 1420, and 905. The factor for the natural oil was 1975.

In the case of Concentrate II, part of it was acetylated and then assayed for vitamin A by both methods. The conversion factor was 1950, very close to that for the oil. Suitable tests were made to show the presence of the ester. In the case of Concentrate III, the vitamin A was obviously in the alcohol form.

These findings would seem to indicate: (1) that the ester form of vitamin A is more easily assimilated by the rat than the alcohol; (2) that vitamin A evidently exists in the natural liver oil chiefly in an ester form; (3) that vitamin A exists in concentrates of liver oils as the ester, as the alcohol, or as a mixture of both forms, depending upon the extent of saponification carried out.

Further work is in progress.

Alcohol Formation in Yeast Grown in Heavy Water. BY E. A. EVANS, JR., AND D. RITTENBERG. *From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

With the aim of using yeast as a source of deuterium-containing physiological substances, and of studying the mechanism of alcoholic fermentation, yeast was grown in a synthetic medium in dilute heavy water. The ethanol formed was isolated, both as ethyl *p*-nitrobenzoate and as ethyl 3,5-dinitrobenzoate, and its deuterium content determined. By oxidation of the alcohol to acetic acid and redetermination of the deuterium content, the distribution of deuterium at each carbon atom of the alcohol could be ascertained. In addition, various constituents of the yeast were isolated and their deuterium content determined.

A New Classification of Carbohydrates. BY MARK R. EVERETT AND FAY SHEPPARD. *From the Department of Biochemistry, University of Oklahoma Medical School, Oklahoma City*

A comprehensive quantitative study of the oxidation of carbohydrates in bromine water has demonstrated that the *cis-trans* interrelation of intermediate cyclic carbons of sugar molecules is one of three general structural influences affecting oxidation. It determines differences in behavior of closely related isomeric sugars, sugar alcohols, and lactones of sugar acids. Carbohydrates can be usefully classified according to their cyclic *cis-trans* isomerism. The pyranoid series, for example, can be arranged in eight *cis-trans* groups, corresponding to the aldopyranopentoses from which they are derived.

We have compared relative interatomic distances in both zigzag and coplanar pyranoid models and have found the average interoxygen distance between adjacent intermediate oxygens 2, 3, and 4 to be greater in *trans* than in *cis* forms, decreasing in the same order of *cis-trans* types found in our oxidation experiments. Evidently the determining interoxygen influences, such as hydrogen bridge formation, orient themselves in *cis* forms between the adjacent oxygen atoms, rather than towards the cyclic oxygen. As Ohle has shown, this affects ring stability. In aqueous solution the equilibrium between furanoid, pyranoid, and ϵ -oxide forms is shifted towards a more stable (*trans*) isomer, with resulting modification of reaction. In our studies, cyclic *trans* isomers were always oxidized more readily than *cis* forms, probably because of increased stability of the *trans* pyranoid ring. The same principle was found to apply to furanoid and ϵ -oxide forms of lactones and heptoses.

The Prolongation of Insulin Action. BY A. M. FISHER AND D. A. SCOTT. *From the Connaught Laboratories, University of Toronto, Toronto, Canada*

It is well known that zinc is a normal constituent of blood and together with spermine has been found in relatively large amounts in normal pancreas. It is shown that the administration of incubated preparations comprising spermine, zinc, and insulin produces prolonged hypoglycemia in rabbits as well as in dogs. A preparation from beef blood is likewise suitable for modifying the blood sugar-lowering effect of the hormone. Zinc is an essential constituent of these modified preparations of insulin. Spermine, zinc, and the blood preparation, occurring as they do in tissues associated with insulin, might be concerned with storage, liberation, or action of the antidiabetic substance in the body. The effects of prolonged heating upon the physical and physiological properties of the modified preparations of insulin are discussed.

The Influence of Fructose on the Glucose Tolerance Curve of Normal and Depancreatized Animals. BY JEAN P. FLETCHER AND E. T. WATERS. *From the Department of Physiology, University of Toronto, Toronto, Canada*

Glucose tolerance curves of normal dogs, and of depancreatized dogs, which have received a suitable subcutaneous injection of protamine zinc insulin, are markedly lowered by the administration of small amounts of fructose. The results throw light on the interpretation of the glucose tolerance curve and on the metabolism of fructose.

Fatty Livers in Geese, Produced by Overfeeding. BY EUNICE FLOCK, JESSE L. BOLLMAN, H. R. HESTER, AND FRANK C. MANN. *From the Division of Experimental Medicine, The Mayo Foundation, Rochester, Minnesota*

Fatty livers were readily produced in geese by continuous overfeeding with a high carbohydrate diet. Values for fatty acids up to 15 per cent were obtained when the geese were fed large quantities of the diet at intervals during the day or moderate quantities both day and night. Values up to 48 per cent were obtained when large quantities were fed day and night. That the fat found in the fatty livers was more saturated than normal was shown by the low iodine numbers. These were generally lower than the

iodine numbers of the depot fat. As the fat increased, the water content of the liver decreased. Glycogen values showed considerable variation.

A large increase in body fat occurred even in the birds which had only moderately fatty livers. In general, an increase in the neutral fat of the blood occurred as the livers became very fatty.

Effect of Deficiencies of Rat and Chick Antidermatitis Factors on Puppies on a Synthetic Diet. BY PAUL J. FOUTS, SAMUEL LEPKOVSKY, O. M. HELMER, AND THOMAS H. JUKES. *From the Lilly Laboratory for Clinical Research, Indianapolis City Hospital, Department of Medicine, Indiana University, Indianapolis, and the Division of Poultry Husbandry, University of California, Berkeley and Davis*

Puppies on purified casein diets supplemented with vitamin B₁ and the various fractions of vitamin B₂ complex have been studied. Puppies on diets supplemented with vitamin B₁, plus flavin, plus rice polish extract containing rat antidermatitis factor but free from flavin and chick antidermatitis factor, and plus liver filtrate ("filtrate factor") containing the chick antidermatitis factor but free from flavin and rat antidermatitis factor, grew at a normal rate. The puppies on the same diet and supplements minus the liver filtrate grew very poorly and died within from 27 to 52 days of black tongue. Those that received neither the rice polish nor the liver filtrate also developed black tongue. The puppies receiving all supplements except the rice polish (rat antidermatitis factor) grew at a subnormal rate and eventually ceased to grow. These dogs were severely constipated and manifested various neurological symptoms including incoordination, involuntary muscle spasms, and generalized convulsions. They died from the effects of severe microcytic hypochromic anemia unless treated with a preparation containing the rat antidermatitis factor.

Further Studies on Factor W. BY D. V. FROST AND C. A. ELVEHJEM. *From the Department of Agricultural Chemistry, University of Wisconsin, Madison*

In a previous paper Elvehjem, Koehn, and Oleson¹² demon-

¹² Elvehjem, C. A., Koehn, C. J., Jr., and Oleson, J. J., *J. Biol. Chem.*, **115**, 707 (1936).

strated the existence of an essential dietary factor distinct from vitamins B₁, B₂, B₄, B₆, and flavins. It was originally designated as the alcohol-ether precipitate factor, but for obvious reasons the term is inadequate and we now suggest the use of factor W until a more complete nomenclature is adopted. Ration K₁₂ described in the earlier paper was used as the basal diet. Young rats placed on this diet and given ample amounts of crystalline vitamin B₁ and 20 micrograms of crystalline flavin per day grew less than 1 gm. per day over a 6 week period. The daily addition of a concentrate of factor W containing less than 1 mg. of solid matter caused a growth of 3.5 to 4.0 gm. per day. Similarly, if factor W is fed without ample flavin, poor growth results until the flavin is added. Concentrates have been prepared from liver extract and milk. Other materials showing high potency are yeast and kidney. Wheat germ, rice bran, and cereal grains in general are low in this factor.

Factor W is inactivated by heating to 100° in acid or alkaline solutions. It is not destroyed by ultraviolet light or irradiation procedures which destroy flavins. The factor as present in partially purified concentrates is not adsorbed on fullers' earth.

The Value of Pyridine Derivatives in Nutrition. BY CASIMIR FUNK AND IAN CASIMIR FUNK. *From Casa Biochemica, Rueil-Malmaison, France*

Nicotinic acid was isolated in 1912 by Suzuki from rice and independently by one of the authors from rice and yeast. The combination of nicotinic acid with two other substances, isolated from the vitamin B complex, resulted in a more prolonged curative effect. Later Szymanska and Funk¹³ studied the nutritional value of some pyridine derivatives, particularly nicotinic acid and nicotinamide, and attributed to them a food-sparing and weight-preserving action. Since the isolation of nicotinamide from heart muscle by R. Kuhn and various pyridine derivatives from enzymes by Warburg and his associates, the subject has received added interest, and therefore the possible nutritive importance of nicotinic acid and nicotinamide was again reinvestigated in three series of animals; namely, (1) pigeons on especially washed rice plus crystalline vitamin B₁; (2) rats on especially purified food

¹³ Szymanska and Funk, *Chem. Zelle u. Gewebe*, **13**, 44 (1926).

ingredients plus crystalline vitamin B₁ and cod liver oil (no other vitamins were added in (1) and (2) to avoid pyridine impurities); (3) rats on artificial dietary plus Vitamin Complex (marketed by the United States Vitamin Corporation).

In all three series of animals nicotinic acid and especially nicotinamide (*per os* or injected) produced a much larger food intake and better weight, as compared with the controls. The experiments will be extended; the authors wish to thank the United States Vitamin Corporation and Dr. Harry E. Dubin for financial support.

The Picrate Precipitate of Serum Ultrafiltrates. BY OLIVER HENRY GAEBLER AND LYNN DEFORREST ABBOTT, JR. *From the Department of Laboratories, Henry Ford Hospital, Detroit*

The precipitate produced in normal ultrafiltrates by picric acid and rubidium chloride at 5°, while small, is quite complex. Uric acid was identified as a major constituent of precipitates from ultrafiltrates of beef serum. Addition of 2 mg. per cent of uric acid, in lithium carbonate solution, to ultrafiltrates of hog serum, made it possible to precipitate the apparent creatinine from these almost as readily as from beef serum ultrafiltrates.

Four lots of precipitate were prepared: two from beef serum and two from hog serum. Each lot contained the equivalent of 30 mg. or more of creatinine. The creatinine content of the hydrochloric acid solutions obtained by decomposing the precipitate with normal hydrochloric acid and ether was essentially the same, whether determined by the picrate or dinitrobenzoate methods. After evaporation of the hydrochloric acid solution, 90 per cent or more of the chromogenic substance could be obtained as a zinc chloride which crystallized on the walls of the tube in rosettes microscopically indistinguishable from creatinine zinc chloride. Analyses are in progress.

A third fraction obtained from the picrate precipitates is insoluble in alcohol and gives the ninhydrin and α -naphthol-hypobromite reactions. It may therefore contain arginine.

Studies of the Perosis-Preventing Properties of Manganese. BY WILLIS D. GALLUP AND L. C. NORRIS. *From the Department of Poultry Husbandry, Cornell University, Ithaca*

Perosis is a bone deformity among certain avian species characterized by swelling of the metatarsal-tibial joint, a slipping of the Achilles tendon from the condyles of the tibia, and a rotation of the metatarsus at the joint. The severity of the condition may be determined by external examination. Either one or both legs of a chick may be affected.

The effectiveness of manganese in preventing perosis was determined with MnCl_2 , MnSO_4 , MnO_2 , MnCO_3 , and KMnO_4 which were added to a basal diet, containing 10 parts per million of manganese, in such amounts as to supply a total of 50 p.p.m. of manganese. Day-old chicks were used as experimental animals.

80 per cent of the chicks on the basal diet developed perosis. Less than 8 per cent of the chicks that received manganese salts developed perosis. MnCO_3 (Mn^{++}) and the relatively insoluble MnO_2 (Mn^{++++}) were as effective as the readily soluble salts, MnCl_2 and MnSO_4 . No perosis developed in the chicks that received the highly oxidized form of manganese, KMnO_4 .

The minimum preventive amount of manganese (as MnCO_3) appears to lie between 35 and 50 p.p.m.; complete prevention, or less than 4 per cent of perosis, was not obtained even with 500 p.p.m. Since these unpreventable cases developed during the first 10 days, the initial stages of perosis may occasionally occur during embryonic growth. Means of insuring an adequate supply of manganese during embryonic development and early growth are being investigated.

The results of the studies conducted thus far provide further proof that manganese possesses certain properties of physiological importance.

The Availability of α -N-Monomethyllysine and α -N-Dimethyllysine for Growth. BY WILLIAM G. GORDON. *From the Department of Chemistry, Stanford University, California*

It has been demonstrated in recent years that the N-monomethyl derivatives of several essential amino acids can be utilized in place of the unsubstituted acids for the stimulation of growth in albino rats maintained on deficient diets. In the present study α -N-monomethyllysine and α -N-dimethyllysine have been synthesized and assayed for their growth-promoting ability as substitutes for lysine.

The methyl lysines were prepared from α -bromo- ϵ -benzoylamino-caproic acid. The bromo acid was treated with aqueous solutions of methylamine and dimethylamine and the benzoyl groups of the resulting compounds were removed by hydrolysis. The final products were isolated in the form of their crystalline, analytically pure monohydrochlorides.

Young albino rats were fed the methyl lysine hydrochlorides incorporated in a lysine-deficient diet in amounts equivalent to 1 per cent *dl*-lysine. Control animals were maintained on the lysine-deficient diet and others were fed this diet supplemented by *dl*-lysine dihydrochloride.

The growth curves of the animals thus obtained show that the rats which had been fed either of the methyl lysines studied grew or declined in weight at substantially the same rate as control animals maintained on the unsupplemented basal ration; the control animals which had been fed *dl*-lysine exhibited rapid growth. It appears, therefore, that neither α -N-monomethyl-lysine nor α -N-dimethyllysine can replace lysine for purposes of growth in the organism of the rat.

Basal Metabolism of Rats on a Phosphorus-Deficient Diet. By HAROLD GOSS AND MAX KLEIBER. *From the Division of Animal Husbandry, College of Agriculture, University of California, Davis*

Basal metabolism measurements were obtained on a number of rats which were fed a diet deficient in phosphorus but otherwise adequate. Control animals were fed upon the same diet to which was added calcium phosphate. Basal metabolism measurements were carried out in a 7 unit, multiple respiration apparatus in which oxygen consumption and carbon dioxide production could be observed over a continuous period of 6 to 12 hours duration.

Growth on the low phosphorus diet was greatly retarded, and appetites were impaired. Daily vaginal smear records showed either long, irregular estrous cycles or complete cessation of the cycle. Although there was considerable variability in the basal metabolic rates, of both experimental and control animals, the mean rate of the high phosphorus group was significantly higher than the mean of the low phosphorus group. However, when the food intake of the controls was restricted to equal that of the

low phosphorus group, it was found that the basal metabolic rate of the phosphorus-deficient group was inclined to be greater than that of the controls, but the difference was not significant.

The results emphasize again the importance of controlling the food intake by paired feeding in metabolism studies on deficient diets. The conclusion that phosphorus deficiency has no significant effect upon the fasting catabolism has been confirmed.

The Euglobulins of Serum and Their Combination with Acid and Base. BY ARDA ALDEN GREEN. *From the Department of Pediatrics, Harvard Medical School, Boston*

The euglobulins of normal horse serum may be separated into at least two fractions by isoelectric precipitation in the absence of salt. This may be accomplished after dialysis of the original serum or after dialysis of an ammonium sulfate precipitate of the globulin fraction.

This finding follows the work in this laboratory on the separation of the globulin fraction of human placental serum and confirms that of Reiner and Reiner¹⁴ on normal and immune horse serum.

The isoelectric points of the two globulin precipitates, P_1 and P_{II} , are in the neighborhood of pH 5 and pH 6, respectively. The total acid and base bound by the two fractions is not the same and the titration curves are markedly different.

Mathematical Relations in the Partition of the Serum Calcium.

BY DAVID M. GREENBERG AND CLARENCE E. LARSON. *From the Division of Biochemistry, University of California Medical School, Berkeley*

The hypothesis that there is an equilibrium between the diffusible and protein-bound calcium of the blood serum which is governed by the mass law has been subjected to a critical study. To obtain data under extreme test conditions, experiments were performed which produced extensive alterations in the content of calcium and the proteins of human and dog blood plasma. Alterations in the plasma proteins were produced by plasmapheresis.

A linear representation of the data was obtained by rearranging the mass law relationship into the form,

¹⁴ Reiner, H. K., and Reiner, L., *J. Biol. Chem.*, **95**, 345 (1932).

$$\frac{P}{CaP} = \frac{1}{A} + \frac{B}{Ca^{++}_D} \quad (1)$$

and plotting P/CaP against $1/Ca^{++}_D$. In Equation 1, P represents the protein concentration, CaP the calcium-protein complex, and Ca^{++}_D the concentration of diffusible calcium. It is assumed that the diffusible calcium is virtually completely ionized. Of the two constants, A represents the maximum calcium-combining capacity per gm. of serum protein. The term B is given by the slope of the straight line. It is equivalent to the equilibrium constant, K_{CaP} , divided by A .

Expressing the protein concentration in per cent and the calcium values in mg. per cent of serum, the best numerical representation of Equation 1 is

$$\frac{P}{CaP} = 0.403 + \frac{5.80}{Ca^{++}_D} \quad (2)$$

From Equation 2 the maximum calcium-combining capacity per gm. of serum protein is calculated as 0.0622 mM, and $10^{-2.44}$ as the mean value of K_{CaP} .

From the evidence available, the reaction between calcium and protein may be represented by the equation $Ca^{++} + P^{-n} = CaP^{-(n-2)}$ where $-n$ represents the number of electronegative charges on a molecule of protein.

Biochemistry of Magnesium Deficiency. BY DAVID M. GREENBERG AND ELMA V. TUFTS. *From the Division of Biochemistry, University of California Medical School, Berkeley*

The course of magnesium deficiency in the rat conforms to two fairly definite phases. The first phase is manifested chiefly by vasodilation, hyperemia, and hyperexcitability; the second by nutritional failure, cachexia, and kidney damage. In animals reared on a diet containing about 1 mg. of Mg per 100 gm. of food, the plasma magnesium begins to decrease by the 3rd day and reaches a minimum level in 7 to 10 days. The red corpuscle magnesium is reduced to about half its normal level during the same period. The magnesium content of the whole animal is reduced, but the soft tissues are nearly unaffected. The calcium content of the carcass and of such tissues as muscle, heart, and kidney is increased.

The second phase is marked by decreased food consumption, retarded growth, rough sticky coat, and finally edema. Chemically the plasma magnesium level shows a sharp rise shortly after the onset of hyperexcitability, after which it again falls off slowly. There are increased urinary excretion of protein and lowered serum protein levels. At the same time, a further decrease occurs in the content of total body magnesium and an increase in the per cent of body calcium. A slight withdrawal of magnesium from muscle and brain is observable. The calcium content of such viscera as heart, muscle, and kidney is greatly increased. Under optimum conditions, the minimum level of magnesium for normal growth is about 5 mg. per 100 gm. of food. A larger amount is required during lactation and with augmented levels of calcium in the diet.

The Synthesis of Crystalline *l*-Cysteinyl-*l*-Cysteine Hydrochloride.

BY JESSE P. GREENSTEIN. *From the Department of Physical Chemistry, Harvard Medical School, Boston*

The behavior on oxidation of molecules containing two or more thiol groups is of considerable biological interest, inasmuch as the physiological characteristics of many proteins are dependent on —SH:—S—S— equilibria. These proteins contain several actual or potential thiol groups. Since our knowledge of the form which the reduced protein assumes on oxidation is incomplete, investigations have been undertaken on simpler, synthetic models containing two or more thiol groups in the hope that they might simulate the more complex behavior of the protein. With this end in view the peptide *l*-cysteinyl-*l*-cysteine hydrochloride was prepared by the following series of reactions: (Cbzo = $C_6H_5CH_2OCO$). $DiCbzo\text{-}cystyl\ Cl + 2\ cysteine\ ethyl\ ester \rightarrow diCbzo\text{-}cystyl\text{-}dicysteinyl\ ethyl\ ester \rightarrow cysteinylcysteine\ ethyl\ ester \rightarrow anhydro\text{-}cysteinylcysteine \rightarrow cysteinylcysteine\ hydrochloride$.

The peptide is formed from the crystalline diketopiperazine by allowing the latter to stand in cold concentrated hydrochloric acid for several days. The peptide hydrochloride separates in about 50 per cent yield in long prisms; m.p. 158° ; $[\alpha]_D^{22} = +44.8^\circ$. For cysteine hydrochloride $[\alpha]_D^{22} = +4.7^\circ$.

When anhydrocysteinylcysteine in aqueous solution is oxidized with $\frac{1}{2}$ hydrogen peroxide, it yields the crystalline dimer, bis-anhydrocystinylcysteine.

Studies Concerning the Production and Cure of Florid Dermatitis (Acrodynia) in the Rat. BY N. B. GUERRANT, FRANCIS CHORNOCK, AND R. ADAMS DUTCHER. *From the Department of Agricultural and Biological Chemistry, Pennsylvania State College, State College*

Since the available literature concerning the production and cure of the florid type of dermatitis in the rat contains many inconsistent results and conflicting conclusions, a series of studies was initiated to determine the effect of such factors as the carbohydrate, fat, and other constituents of the diet on the production of this nutritional disorder and the relative efficiencies of certain dietary substances in curing it.

The florid type of dermatitis was produced consistently in rats receiving diets containing glucose or sucrose as the source of carbohydrate and to a variable degree in rats receiving diets containing corn-starch or rice starch. This form of dermatitis was also quite regularly produced in rats receiving diets containing 10 per cent of lard or 20 per cent hydrogenated cottonseed oil, and less regularly in rats receiving a similar diet containing 8 per cent of filtered butter fat. Further purification of the casein beyond that usually employed in our vitamin B complex-deficient diets did not hasten the onset of the syndrome. Increasing the vitamin B₁ intake beyond 8 micrograms per day brought about an earlier death, while increasing the lactoflavin intake up to 16 micrograms per day had no effect on length of life but did appear to amplify the dermatitis. Yeast, ether-extracted yeast, white corn-meal, corn oil, wheat germ oil, and linseed oil were effective in curing this form of dermatitis. Of the oils tested, wheat germ oil proved most effective.

The Quantitative Determination of Estrogenic Substances in Normal Female Urine during the Menstrual Cycle. BY R. G. GUSTAVSON, EDWIN E. HAYS, AND THOMAS R. WOOD. *From the Research Laboratories of the University of Denver, Denver*

24 hour and 12 hour samples of urine from normal women have been analyzed for estrogens. The urine after hydrolysis by boiling for a 2 hour period at approximately pH 0.7 was extracted with

chloroform for 24 hours. This extract was assayed by the vaginal smear method on spayed rats, twenty to thirty rats for the assay of each day's excretion. Two peaks of excretion have been found consistently. The maximum excretion has been found to be equivalent to 90 micrograms of theelin (estrone) for a single day. A portion of each day's extract was reserved for a composite sample. The total excretion from an analysis of this composite during a menstrual cycle was equivalent to 1.3 mg. of theelin. Summation of the analysis of the daily samples gave 0.94 mg. Night and day excretion was compared in one individual.

The first peak of excretion may be coincident with ovulation. For a varying period of time following rupture, during the time of hemorrhage into it, and before the migration and growth into it of the lutein cells, one would expect a drop in estrin production. While the corpus luteum is responsible for the formation of progesterone, it also continues to form estrin to as great an extent, if not greater, than the follicle. This would account for the second estrin peak. The regression of the corpus luteum, with a diminution or disappearance of estrin, is followed shortly by menstruation.

Attempts to Isolate the Anti-Egg Injury Factor (Vitamin H)*. By PAUL GYÖRGY. *From the Babies and Childrens Hospital, and the Department of Pediatrics, School of Medicine, Western Reserve University, Cleveland*

In its natural state this factor, termed vitamin H by the author (1931), is neither water- nor fat-soluble. Its water solutions can be prepared from liver only after proteolytic digestion with papain or after hydrolysis under high pressure with low acidity, and from yeast after autolysis in the presence of toluene (Hudson) but not of chloroform, or by a combination of chloroform and consecutive toluene autolyses.

By these methods water-soluble concentrates suitable for purification were first obtained from the dried residue of the liver preparation campolon. After charcoal adsorption, elution with pyridine-methanol, boiling with sulfuric acid, and subsequent precipitation with alcohol, concentrates were obtained from

* This work was done partly in collaboration with R. Kuhn, E. Lederer, F. Schultz, and T. W. Birch.

which vitamin H could not longer be precipitated by phosphotungstic acid or gold chloride. By a combination of these methods the unit of vitamin H was reduced from 0.1 gm. of liver residue to 0.3 to 0.5 mg. of concentrate. It is 3 to 5 times as effective parenterally as *per os*.

When such concentrates are evaporated to dryness, extracted with glacial acetic acid, and precipitated with ethyl acetate, vitamin H remains in solution. By ether and ethyl acetate precipitation the unit has been lowered to 10 to 20 micrograms (parenteral). The barium salt of vitamin H is not ethanol-soluble; the sodium salt is readily soluble in absolute alcohol; concentrates active in 3 to 5 micrograms were thus obtained. In further purification vitamin H is remarkably unstable. On electrodialysis it exhibits distinct acid character. Precipitation with different alkaloids was not successful. Treatment with nitrous acid, ketene, formaldehyde, and benzoyl chloride inactivated vitamin H. Vitamin H appears to be an ampholyte and in particular an acidic amino acid.

***d*-Lysine and Growth.** BY FREDERICK S. HAMMETT. *From the Laboratory of the Marine Experimental Station of the Lankenau Hospital Research Institute, North Truro, Massachusetts*

108 experiments with more than 50,000 animals (*Obelia geniculata*), half of which were exposed to *d*-lysine in concentrations ranging from $m/1,000,000$ to $m/6250$, show that this amino acid is not a stimulus to growth as a whole. Although proliferation expression is advanced in its presence and differentiation expression held back, these responses are not specific to lysine. They have been produced by other nucleoprotein components. Where lysine differs from other compounds is in the production of an increased regression of the complete hydranths at the very low and non-toxic concentration of $m/1,000,000$. This fact and correlated data from these and other experiments justify the conclusion that the compound is essential to the mass increment or anabolic phase of growth, and this, not as a direct stimulus thereto, but as a specific participant in some enabling process therefor.

The Speed with Which Various Parts of the Body Reach Equilibrium in the Storage of Alcohol. BY R. N. HARGER, H. R.

HULPIEU, AND E. B. LAMB. *From the Department of Biochemistry and Pharmacology, Indiana University School of Medicine, Indianapolis*

Determinations were made of the alcohol content of the alimentary tract, brain, blood, liver, and muscle of dogs that had received alcohol by mouth or intravenously and were killed at various intervals. At the end of 3 hours, both methods of administration produced practically the same alcohol concentration in the alimentary tract (about 5 per cent of the total body alcohol) and this equilibrium was still maintained after 12 hours. The ratios of alcohol concentrations in brain, blood, and liver were quite constant for all intervals studied, while the concentration of muscle alcohol showed a distinct lag during the 1st hour. The unabsorbed alcohol, following oral administration, when calculated as excess above equilibrium values for the alimentary tract, averaged 47.7 per cent at 15 minutes, and 1.0 per cent at 3 hours. After equilibrium was established, all of the organs studied, including the alimentary tract, stored alcohol in about the same proportions as their water content, average alcohol ratios on this basis being (brain = 1.00) blood 1.18 ± 0.08 , liver 0.95 ± 0.04 , muscle 1.01 ± 0.04 , stomach contents 1.13 ± 0.08 , stomach tissue 0.93 ± 0.06 , and intestine 0.98 ± 0.04 . Determinations of alcohol in blood and spinal fluid of 50 human cases from the emergency ward of a hospital gave an average ratio of spinal fluid to blood of 1.19 ± 0.09 . Brain alcohol may be predicted with reasonable accuracy from the alcohol content of blood as well as from that of other parts of the body.

The Antirachitic Property of Casein. BY ROBERT S. HARRIS AND JOHN W. M. BUNKER. *From the Biological Research Laboratories, Massachusetts Institute of Technology, Cambridge*

It has not been possible to produce experimental rickets in rats with diets in which casein from cow's milk serves as the protein constituent, even when the ratio of calcium to phosphorus in the diet is 9:1, when the phosphorus content of the diet is less than 0.20 per cent, and when extractable fat-soluble factors are removed from the diet. Partially dephosphorized casein retains its anti-rachitic property, while caseins which have been dephosphorized rather completely are toxic to rats. Digestion with trypsin does

not alter the antirachitic property of casein and increases the solubility of casein in water.

Concentration of a Hyperglycemic Factor from Urine. BY BENJAMIN HARROW, ABRAHAM MAZUR, I. M. CHAMELIN, AND ALEX LESUK. *From the Chemical Laboratory, the City College, College of the City of New York, New York*

By dialysis and removal of inorganic salts, the crude material, with an activity of 1.1 units per gm., has been concentrated to give an activity of 83 units per gm. 1000 liters of male urine yield, approximately, 200 mg. of the purified material.

The Toxicity of Calciferol for Rabbits. BY ARTHUR M. HARTMAN. *From the Division of Nutrition and Physiology, Bureau of Dairy Industry, United States Department of Agriculture, Beltsville*

Rabbits, weighing approximately 1.0 to 1.5 kilos, were weaned at about 60 days of age and placed upon diets of natural foods consisting of 20 gm. daily of grain, and alfalfa hay of either poor quality (A_2) or good quality (A_1), *ad libitum*. To groups of these animals were administered 6 days per week by mouth various doses of calciferol dissolved in 1 gm. of cottonseed oil.

The survival periods of rabbits on A_2 hay and calciferol decreased on the average with increasing dose at levels below 2.0 mg. daily. As evidenced by changes in body weight and by survival period, the data indicate that the toxicity is antagonized by alfalfa hay of good quality. The data also suggest that calciferol may be more toxic for males than for females. Autopsy and histological examination of a few tissues disclosed extensive degeneration and calcification of kidney tissue or calcification of the aorta, or both, in one or more animals in each of the calciferol-fed groups, even when the daily dose of calciferol was as small as 0.02 mg. These results indicate that moderate doses of calciferol which do not kill rabbits for periods up to 8 months may nevertheless produce degeneration and calcification in the tissues and organs.

Spectrophotometric tests indicated that the calciferol was pure when obtained and was not altered in the experimental handling.

Physiological Effects of Phenol-Contaminated Drinking Waters. BY V. G. HELLER AND LEE PURSELL. *From the Oklahoma Agricultural Experiment Station, Stillwater*

Phenol has long been considered a germicidal and toxic compound. As an industrial waste and decomposition end-product, it may often contaminate drinking waters and produce undesirable tastes, especially when chlorine and certain other organic compounds are present. Biological tests have been made with rats to determine whether such waters were toxic when used as a constant source of drinking supply. It is surprising that waters containing 5000 to 8000 parts per million apparently do not interfere with growth, reproduction, and lactation, and that even 12,000 parts per million may be tolerated. Careful records of food intake, water consumption, and a study of the coefficients of apparent digestibility and nitrogen balances indicate that normal metabolism is not disturbed until the concentration approaches 10,000 parts per million. From quantitative determinations of the free and total phenolic compounds in the feed, feces, urine, and blood the phenol balances and the paths and forms of excretion were determined. It is apparent that the phenolic compounds are detoxicated by being converted into conjugate forms which are rapidly eliminated in the urine. Some phenolic compounds are lost and they are probably excreted in the urine in forms not detected by the present reagents. Tisdall's method was used for the determination of phenolic compounds in the urine. Rakestraw's method was used in the blood studies. Existing methods for the analysis of feed and feces were found to be unsatisfactory, since they are too inclusive; therefore, a modified Tisdall procedure was devised and applied.

Further Studies on the Effect of Aldehydes on Cystine and Cysteine. BY W. C. HESS AND M. X. SULLIVAN. *From the Chemo-Medical Research Institute, Georgetown University, Washington*

At the 1935 New York meeting of the American Chemical Society, Division of Biological Chemistry, it was shown by Hess that aldehydes had little effect on cystine or cysteine estimation by the Sullivan or Okuda method when 3 moles of aldehyde were added to 1 mole of cystine or cysteine in 0.1 N HCl. In further work formaldehyde was placed in contact with cystine and cysteine at various degrees of acidity for varying lengths of time. Upon the cystine determination the aldehydes had little effect, whether in dilute or relatively concentrated solution. Upon

cysteine they had a marked effect, depending on (1) the degree of acidity, (2) length of contact, (3) molecular proportions, and (4) concentration. In relatively strong solution, the cysteine and formaldehyde speedily combine to form thiazolidinecarboxylic acid as shown by Ratner and Clarke.¹⁵ The degree of such new formation depends on the factors mentioned above and the Sullivan cysteine reaction becomes progressively negative. This phenomenon obtains to only a slight degree in dilute solution (1 mg. of cysteine in 5 cc. of solution) at pH 1.2 and 3.

Further Studies on the Absorption, Excretion, and Mode of Action of Vitamin D. BY WALTER HEYMANN. *From the Babies and Childrens Hospital, and the Department of Pediatrics, School of Medicine, Western Reserve University, Cleveland*

The antirachitic efficacy of intramuscularly injected vitamin D decreased considerably in rachitic rats in which biliary cirrhosis or degenerative changes of the liver had been produced. The jaundice did not interfere with calcification, because administration of phosphate solution produced calcification in the jaundiced rachitic rats as easily as in the controls. Impaired liver function therefore seemed to be responsible for the decreased antirachitic efficacy of vitamin D, a hypothesis first advanced by Gerstenberger.

In view of these results it seemed advisable not to depend on the incurability of rickets as the criterion for an absorption failure of vitamin D, but to administer vitamin D orally and then to test its presence in the blood of dogs with and without bile obstruction. Accordingly the serum of these dogs was injected into rachitic rats, and it was found that vitamin D, whether given as viosterol or as the water-soluble preparation drisdol, was not present in the blood of dogs in which jaundice had been induced by ligation of the common bile duct. This direct evidence definitely proves that vitamin D is not absorbed unless bile is present in the chyme. The excretion of vitamin D through the intestinal wall and through bile was also studied in dogs which had been subjected to bile duct obstruction and which had received vitamin D by intramuscular injection. The results of these experiments definitely

¹⁵ Ratner, S., and Clarke, H. T., *J. Am. Chem. Soc.*, **59**, 200 (1937).

prove that vitamin D is reexcreted through the wall of the small intestine as well as through the wall of the large intestine.

Oxygen Uptake by Dried Hemoglobin. BY ALAN HISEY. *From the Department of Chemistry, University of Tennessee School of Biological Sciences, Memphis*

The oxygen uptake by samples of dried, reduced hemoglobin has been studied in Warburg microrespirometers at a temperature of 37.5°, the gas phase being either air or oxygen. Observations were made simultaneously on three to six samples of each hemoglobin preparation. At varying intervals individual samples were analyzed spectrophotometrically for methemoglobin and active and total pigment.

There is an initial rapid oxygen uptake; the rate gradually falls off, but oxygen consumption is still evident after 180 hours. In pure oxygen both the rate and amount of oxygen consumed are larger than in air.

Although methemoglobin appears to be the principal, if not the only end-product, the volume of oxygen utilized is larger than would be required for this conversion on the basis of 0.25 molecule of oxygen for each atom of iron oxidized. In the presence of pure oxygen the volume change is larger than that which would be calculated for the conversion to oxyhemoglobin. Since there is no indication of carbon dioxide production, part of the oxygen which disappears is probably adsorbed.

The uptake of other gases by dried hemoglobin is being studied.

Molecular Constitution of Tooth and Bone Phosphates. BY HAROLD CARPENTER HODGE, WILLIAM F. BALE, AND MARIAN L. LEFEVRE. *From the Department of Biochemistry and Pharmacology and the Department of Medicine, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*

The problem of the molecular constitution of the principal inorganic component of bone and tooth substance has been approached by chemical methods (analytical, synthetic, electrometric) and by x-ray diffraction studies. Teeth, bones, commercial tricalcium phosphates, and calcium phosphates precipitated at pH 7, all have hydroxylapatite diffraction patterns before ignition. After ignition, more or less change occurs to β - $\text{Ca}_3\text{P}_2\text{O}_8$ patterns, depending

on the Ca:P ratio. At pH 7, the Ca:P ratio depends more on mode of precipitation than on amounts of Ca and P added. From electrometric titrations, the precipitates are either CaHPO_4 or have the hydroxylapatite lattice.

A Photometric Method for the Determination of Potassium in Minute Amounts of Serum. BY WILLIAM S. HOFFMAN. *From the Department of Physiological Chemistry, Chicago Medical School, Chicago*

The colorimetric method of Jacobs and Hoffman for the determination of potassium has been adapted to the Cenco-Sheard-Sanford photelometer. Potassium is precipitated as $\text{K}_2\text{NaCo}(\text{NO}_2)_6$ and the cobalt is determined by measuring the emerald-green color produced on adding choline and ferrocyanide. When these colors are determined in the photelometer with a blue filter, the microammeter readings obtained give smooth curves when plotted on semilogarithmic paper. The curves for 1 cc. and 0.5 cc. of serum are somewhat concave, but that for 0.2 cc. is practically a straight line for the range of values found in serum. The equation of this curve is $C = (239.8 \pm 4.8) \log 86.0/R$, where C is the concentration of potassium in serum and R is the microammeter reading. This curve has enough slope to permit determination of 0.2 cc. of serum with a degree of accuracy greater than for 1 cc. with the ordinary colorimeter. The technique of precipitation and washing has been modified to permit direct determinations on old serum which have hitherto been unreliable. A correction factor has been introduced to allow for possible changes in the blank, as ferrocyanide is slowly oxidized to the more highly colored ferricyanide.

A New Deficiency Disease Associated with the Vitamin B Complex. BY ALBERT G. HOGAN, LUTHER R. RICHARDSON, AND PAUL E. JOHNSON. *From the Department of Agricultural Chemistry, University of Missouri, Columbia*

The basal diet (casein 20, sucrose 71, salts 4, cellulose 3, cod liver oil 2) is supplied to pigeons until they develop polyneuritis or until they lose 30 per cent of their normal weight. If a highly concentrated antineuritic preparation is administered at this point, the pigeons recover from polyneuritis but they do not regain nor-

mal weight, and, if this regimen is continued, the pigeons become severely anemic in about 9 weeks and soon die. The red cell counts may fall below 1 million per c.mm. Neither flavin nor an antidermatitis concentrate has any effect on erythrocyte regeneration. Attempts to prepare antianemic concentrates that are free from other vitamins have not been successful.

As anemia develops, a considerable proportion of the red cells becomes abnormal, with a characteristic sickle form. During the last stage of anemia half of the red cells may be deformed, and if a curative agent is then administered their number increases rapidly at first. After about 10 days the number decreases and practically all will disappear by the end of the 5th week.

The anemia is not due to fasting, for if anemic pigeons are given a complete diet, but restricted in quantity so as to prevent gains in weight, the red blood cells become normal in appearance and in number. This type of anemia is therefore regarded as a hitherto unrecognized deficiency disease.

The Effects of Minute Amounts of Lead on the Animal Organism.

BY M. K. HORWITT AND GEORGE R. COWGILL. *From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*

According to previously accepted standards small amounts of lead in the diet have a deleterious effect on the animal organism. However, this view has never been adequately tested.

A new titrametric dithizone technique useful for microanalyses for lead was developed. Rats were fed diets containing from less than 1 to more than 100 mg. per kilo of food. Diets containing up to 100 mg. per kilo did not affect growth. In very young rats slight stunting occurred with foods containing 200 mg. per kilo but recovery was complete after the 4th month. No fall in blood hemoglobin was obtained until more than 200 mg. per kilo of diet were ingested. A few cases of "stippling" were found in the blood of young rats on regimens containing more than 100 mg. per kilo but this disappeared after the 4th month. In a reproduction study three generations of normal rats were successfully reared on diets containing up to 100 mg. per kilo. Blood and bone analyses were also made. Many experimental diets in common use in both this and other laboratories were found to

contain more than the present government tolerance level of 2.6 mg. per kilo of food, and this without adversely affecting the animals subsisting thereon.

Several litters of puppies were also raised on artificial diets with added varying amounts of lead and with no deleterious effects from diets containing 50 mg. per kilo. The effect of lead absorbed from different cages was also investigated.

Is Skeletal Maturity Related to Calcium Storage in Children? BY HELEN A. HUNSCHER, FRANCES COPE HUMMEL, ICIE G. MACY, T. WINGATE TODD, AND C. C. FRANCIS. *From the Research Laboratory of the Children's Fund of Michigan, Detroit, and from Western Reserve University, Cleveland*

Ten children (5 to 8 years) have been observed in a metabolic study over 8 continuous months. The following criteria served as the basis for the selection of the subjects: a detailed medical history showing an absence of clinical signs of health defects and a record of growth progress attained by anthropometric measurements and roentgenological study of the skeleton. To aid in the standardization and avoid possible unfilled calcium stores in their bodies, 1 quart of milk was consumed daily by each child for 3 months preceding the metabolic collections.

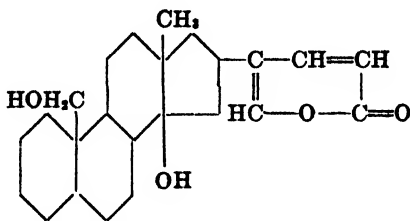
The range of differences between the chronological age and skeletal maturity (Todd method) amounted to 30 months at the beginning of the study in October and was unchanged in March.

In this longitudinal study of children the total accumulation of calcium showed wide variations irrespective of diet, which became less as the study progressed. During the successive months the range of daily average calcium retention per month for all the children was 0.214, 0.100, 0.113, 0.122, 0.079, and 0.058 gm. respectively.

Chemical Studies on Toad Poisons. Further Contributions to the Chemical Constitution of Marinobufagin, Cinobufagin, and Gamabufagin. BY H. JENSEN. *From the Laboratory for Endocrine Research, the Johns Hopkins University, School of Medicine, Baltimore*

Marinobufagin, $C_{24}H_{32}O_6$, and cinobufagin, $C_{22}H_{34}O_6$, are unsaturated hydroxy lactones, cinobufagin being the acetyl deriva-

tive of the compound $C_{24}H_{32}O_5$. Tschesche and Offe¹⁶ and independently Jensen¹⁷ showed that cinobufagin on dehydration with selenium yields the Diels hydrocarbon, which marinobufagin also yields, indicating the presence of the sterol ring structure in both. Repetition of catalytic hydrogenation has revealed the presence of three double bonds instead of two, as had previously been reported¹⁸. Tschesche and Offe¹⁹ have since reported similar findings. Ozonation of marinobufagin and of cinobufagin yields formic and glyoxylic acids, indicating that two of these double bonds are in the lactone group, a 6-membered ring identical with that in scillaren. The third double bond is in the sterol ring. Marinobufagin and gamabufagin contain a $—CH_2OH$ group attached at C_{10} or C_{13} , corresponding to one of the quarternary methyl groups of the sterols. By strong acid or alkali, this primary alcoholic group can be eliminated as formaldehyde. On oxidation, marinobufagin gives an aldehyde. A tertiary hydroxyl group is attached at C_{14} in all three principles. Whether the third hydroxy group in marinobufagin is also tertiary is uncertain, as is its position. The following structure for bufagin is suggested.



Analytical data obtained for gamabufagin, acetylgamabufagin, and anhydrogamabufagin indicate the empirical constitution of $C_{24}H_{34}O_5$ for gamabufagin (as suggested by Wieland and Vocke²⁰). Gamabufagin contains only two double bonds, both in the lactone ring. Under the influence of acid, gamabufagin loses 1 molecule

¹⁶ Tschesche, R., and Offe, H., *Ber. chem. Ges.*, **68**, 1998 (1935).

¹⁷ Jensen, H., *J. Am. Chem. Soc.*, **57**, 2733 (1935).

¹⁸ Jensen, H., and Evans, E. A., Jr., *J. Biol. Chem.*, **104**, 307 (1934).

¹⁹ Tschesche, R., and Offe, H., *Ber. chem. Ges.*, **69**, 2361 (1936).

²⁰ Wieland, H., and Vocke, F., *Ann. Chem.*, **481**, 215 (1930)

of water. Both cinobufagin and gamabufagin have a structure similar to that of marinobufagin.

Lactate and Pyruvate in Blood and Urine after Exercise. BY ROBERT E. JOHNSON AND H. T. EDWARDS. *From the Fatigue Laboratory, Morgan Hall, Harvard University, Boston*

Current schemes for anaerobic glycolysis in muscle suggest that if the source of the excess lactate found in the body after exercise is the musculature, the concentrations of lactate and various other substances in the body after exercise should vary together. Pyruvate is commonly regarded as the immediate precursor of lactate in muscle glycolysis. We have, therefore, estimated lactate and pyruvate in the blood and urine of young men after hard running.

The lactate and pyruvate recovery curves for blood had similar shapes, though the pyruvate present in any given sample amounted to about one-thirtieth of the lactate. The pyruvate maximum came later than the lactate, but both curves fell off in the same way. The shapes of the curves for urine were remarkably similar, excretion of both excess lactate and pyruvate being complete in about 40 minutes. Here again the amount of pyruvate in relation to lactate was small, the ratio of lactate to pyruvate being 60:200. We isolated pyruvic acid as the 2,4-dinitrophenyl-hydrazone from blood and urine collected after hard running.

These facts seem to support the validity of the Embden-Meyerhof scheme for muscle glycolysis *in vivo*.

The Storage of Carbohydrate by the Liver during Undernutrition.

BY MARGARET WOODWELL JOHNSTON AND L. H. NEWBURGH. *From the Department of Internal Medicine, University of Michigan, Ann Arbor*

It is generally believed that an individual who ingests a diet that is below his maintenance requirement will oxidize all the carbohydrate of the diet plus any which he may be able to release from his body stores. Individuals who have been subjected to varying degrees of undernutrition and varying amounts of dietary carbohydrate have been studied in the respiration chamber, which employs the open circuit principle of indirect calorimetry. Their total heat production and the carbohydrate oxidized in single 24

hour periods and in several consecutive 24 hour periods have been determined. In these experiments it has been repeatedly observed that normal individuals may fail to oxidize all the carbohydrate of their diet even though they are existing on a diet low in carbohydrate and significantly below their caloric requirement. For example, a young normal male, after being fed for 2 days a diet close to maintenance which consisted of 3500 calories and 265 gm. of carbohydrate, was then studied in the respiration chamber for two consecutive 24 hour periods. In the first period he fasted and in this 24 hours oxidized 146 gm. of carbohydrate. In the second period he ate a diet which contained 97 gm. of carbohydrate and yielded 1518 calories. In this 24 hours he oxidized but 53 gm. of carbohydrate, indicating a storage of 44 gm., even though his total heat production was 2236 calories.

The ability of the liver to store glycogen under these conditions is believed to be related to its degree of depletion.

The Rate at Which Glucose Enters the Duodenum from the Stomach. BY WALTER G. KARR, J. HAROLD AUSTIN, W. O. ABBOTT, AND O. D. HOFFMAN. *From the Gastro-Intestinal Section of the Medical Clinic and the Department of Research Medicine, University of Pennsylvania, Philadelphia*

Since a multilumened tube (Miller and Abbott) is now available whereby it is possible to isolate a segment and collect specimens from any part of the small intestine of man, an extended study was planned to determine the response of the gastrointestinal tract to glucose from the time it enters the stomach until it is absorbed. This investigation has been divided into three parts dealing with the factors which determine the character of the responses in (a) the stomach, (b) the duodenum, (c) the jejunum and ileum.

In work to be reported elsewhere it is shown that what occurs in the duodenum is dependent on the amount and concentration of the glucose which is received from the stomach; also that these factors are controlled not so much by the activity of the pylorus as by the amount and concentration of glucose which is introduced into the stomach. The present paper is concerned only with the response of the stomach.

In about 90 experiments various volumes and concentrations of glucose were introduced into the stomach by tube. Often in

addition a multilumened tube had previously been introduced into the intestine. After 15 to 60 minutes the stomach was emptied and washed. The residue obtained was analyzed for glucose and the amount leaving the stomach determined by difference.

From these data numerous correlations were made between the amount of glucose leaving the stomach and the factors: volume, concentration, and total amount of glucose administered.

Some Observations on the Determination of Cystine and Methionine in Proteins. BY BEATRICE KASSELL. *From the Department of Chemistry, New York State Psychiatric Institute and Hospital, New York*

The following determinations were carried out on various proteins and amino acids: total sulfur (Pregl, Parr bomb), inorganic sulfate (BaSO_4 , Baernstein), cystine (Folin photometric, Sullivan, Baernstein), methionine (Baernstein, volatile iodide, homocysteine). On the basis of a series of such determinations it is possible to ascertain the cystine and methionine content of a protein with an accuracy of about 2 per cent.

Certain small corrections have to be applied to Baernstein's methods; digestion with HI results in some formation of H_2S from cystine and of H_2S and methyl mercaptan from methionine. The cystine in certain proteins (*e.g.* insulin) may yield H_2S during the HI digestion to a greater extent than free cystine. The lability of cystine derivatives towards HI is under investigation.

Hydrolysis for the determination of cystine by the Folin photometric and the Sullivan methods was carried out in an oil bath at 130° for varying periods of time and with a number of different acids. Prolonged hydrolysis and particularly the use of H_2SO_4 may result not only in cystine destruction, but also in the formation of substances interfering with the Sullivan reaction.

Under optimum conditions, which may vary for different proteins, the values for cystine (including traces of cysteine) obtained by the Sullivan, Folin photometric, and Baernstein methods are in close agreement. Within the limits of error, cystine and methionine, in the proteins so far investigated, account for the total organic sulfur.

Further Investigation of the Suprarenal Cortex. BY EDWARD C. KENDALL, HAROLD L. MASON, W. M. HOEHN, AND BERNARD .

F. MCKENZIE. *From the Section on Biochemistry, The Mayo Foundation, Rochester, Minnesota*

Compounds A and B (B is identical with Reichstein's corticosterone) are $C_{21}H_{32}O_4$ and $C_{21}H_{30}O_4$ respectively—the O_4 series. Compounds E, F, G, and C are $C_{21}H_{32}O_5$, $C_{21}H_{30}O_5$, $C_{21}H_{32}O_5$, and $C_{21}H_{34}O_5$ respectively—the O_5 series. Compound B has slightly more physiologic activity than A (Ingle's rat test). The combined activity of Compounds A and B is less than 20 per cent of the total activity. In oil Compound E has activity of the same order as A. Compounds A, B, and E contain sterol ring structure with a ketone group at C_3 and a double bond 4,5. If the double bond 4,5 is reduced with the rest of the molecule unchanged, physiologic activity is destroyed.

Compounds A and B are converted with periodic acid into Acid 1, $C_{20}H_{28}O_4$ and Acid 2, $C_{20}H_{26}O_4$ respectively, and formaldehyde. Periodic acid oxidizes Compound E to Acid 5, $C_{20}H_{26}O_5$, and formaldehyde. The 2-carbon chain in Compounds A, B, C, E, F, and G is an α -ketol. Chromic acid oxidizes Acid 2 to Acid 1, indicating conversion of a secondary alcohol into a ketone. This ketone group in Acid 1 and the corresponding hydroxyl group in Acid 2 are provisionally placed on carbon atom 12. Chromic acid converts Acid 5 into a monohydroxydiketone, $C_{19}H_{24}O_5$, indicating an OH group on C_{17} which is converted to a ketone group with loss of CO_2 . Acid 5 is probably identical with Acid 2 except for the additional OH group on C_{17} and a second double bond not yet placed. The most physiologically active fraction remains in solution after separation of Compounds A, B, E, and six other crystalline compounds, all of which differ from A and E only in degree of saturation and number of ketone groups. Distribution of the most active fraction, which is at least 10 times more active than Compound B, between water and benzene, indicates that it belongs to the O_5 series of compounds with OH on C_{17} .

The Nutritive Value of Lactalbumin. BY M. C. KIK. *From the Department of Agricultural Chemistry, University of Arkansas, Fayetteville*

In a comparative study on the nutritive value of lactalbumin in which casein was used as a standard of comparison it was found that 8 per cent lactalbumin had a higher nutritive value than 8 per cent casein. The average gain in weight per gm. of protein

fed was 2.17 for six animals fed lactalbumin and 1.66 for three rats fed casein.

It was found that higher nitrogen retention had taken place in the animals receiving lactalbumin. When the retention is expressed in per cent of the nitrogen intake, these values for six lactalbumin-fed animals were 39, 34, 44, 39, 36, and 28; and for three casein-fed rats, 15, 36, and 26.

A total of twenty-one rats was used in metabolism studies for the determination of the biological value. Casein was found to have a biological value of 70, casein supplemented with cystine 83, lactalbumin 87, and lactalbumin with cystine 87.

The nutritive value of lactalbumin and casein was further compared by the modified, paired feeding method, with nine pairs of rats. The intake of food and the gain in weight were the same for all pair mates, but the intake of nitrogen was lower for the lactalbumin-fed animals. 1 gm. of lactalbumin was more effective than 1 gmp. of casein in promoting maintenance and growth.

Improvement and Standardization of Rat Growth Technique for Short Period Assays of Vitamin B. BY ELIZABETH M. KNOTT AND FREDERIC W. SCHLUTZ. *From the Department of Pediatrics of the University of Chicago, Chicago*

Results 100 per cent higher were obtained in assaying Merck's crystalline vitamin B if the basic ration contained autoclaved liver instead of autoclaved whey as a source of the vitamin G complex. On the liver ration 1.25 micrograms gave 1 gm. of growth in contrast to 2.55 micrograms for autoclaved whey. The difference was not due to residual vitamin B in the autoclaved liver, since negative control rats died with polyneuritis at an average of 36.6 days for whey and 38.0 days for liver. When autoclaved whey was supplemented with Elvehjem's alcohol-ether precipitate factor of liver extract, assay results approached values obtained with autoclaved whole liver.

The amount of international standard adsorbate required for a gm. of growth was 6.7 mg. on the whey ration and 5.4 mg. on the liver ration. Since these results differ by 24 per cent rather than 100 per cent, it would appear that international standard

adsorbate furnished in addition to vitamin B some of the vitamin G complex which was deficient in the whey ration.

10 day assay periods with the liver ration afford a reliable technique for vitamin B, since stabilized wheat germ fed to nine groups of rats at levels ranging from 0.05 to 0.3 gm. per day gave unit values for 1 gm. of growth ranging only from 0.054 to 0.068 gm.

The Effect of Epinephrine Injected Intravenously at a Constant Rate in Normal and Hypertensive Cases. BY ALFRED E. KOEHLER, NORMAN MARSH, AND ELSIE HILL. *From the Santa Barbara Cottage Hospital and the Sansum Clinic, Santa Barbara*

Epinephrine (1:50,000) was injected intravenously from a pump at a uniform rate of 2 mg. per hour. This produced a constant, sustained rise of systolic blood pressure in the normal (average of seventeen cases) of 51 mm. of Hg. The diastolic pressure during the first period rose but then usually dropped well below the control period. With this drop in diastolic pressure there usually was a rise in skin temperature and an increase in venous blood oxygen saturation.

In the normal, at the end of an hour, the average increase in the basal rate was 29 per cent, in blood sugar 141 mg. per cent, and in blood lactic acid 22.3 mg. per cent.

After the injection was stopped, the systolic and diastolic pressures markedly dropped in a few minutes to values well below the initial control period. Concurrently with this drop there was a further and more marked rise in skin temperature and a further increase in the venous blood oxygen saturation, the latter frequently to arterial level.

In essential hypertension (fourteen cases) the findings were not grossly different from the normal responses in regard to basal rate and blood sugar, but there was a lessened increase in the blood lactic acid. The hypertensive group differed markedly, however, in registering a greater pressor effect. There was particularly an increased drop and duration of drop in the blood pressures after the injection. Systolic pressures of 85 were not unusual for short periods. There was no evidence that myocardial failure was the cause of the low pressures.

The Significance of Carbon Dioxide Tension for Metabolic Stimulation by Substituted Phenols. BY M. E. KRAHL AND G. H. A. CLOWES. *From the Lilly Research Laboratories, Indianapolis*

If oxygen consumption of fertilized sea urchin (*Arbacia punctulata*) eggs is plotted against the logarithm of the concentration of the 4,6-dinitro-*o*-cresol, the curve rises nearly linearly until an optimum concentration is reached; beyond this point the curve falls, at first linearly, and then more slowly, nearly to zero.

As the partial pressure of CO₂ is increased, the position of the rising portion of this curve is essentially unchanged; stimulation obtained with high concentrations of the reagent becomes less. The falling portion of the curve therefore sets in at a lower concentration of phenol reagent than in the absence of CO₂. Hence the optimum concentration and the number of units of excess oxygen consumed at the optimum decrease as the CO₂ partial pressure increases. Since the oxygen consumption of eggs not treated with the substituted phenol is inhibited to a marked degree by CO₂, the percentage stimulation by suboptimal concentrations of 4,6-dinitro-*o*-cresol increases as CO₂ partial pressure rises.

The R.Q. of eggs untreated with 4,6-dinitro-*o*-cresol is 0.90 to 0.95 in air and falls to 0.75 to 0.80 as the CO₂ partial pressure rises. Addition of 4,6-dinitro-*o*-cresol at optimum concentration restores the R.Q. to a value approximating unity.

In an atmosphere containing CO₂, sea urchin eggs have a significant aerobic acid production. Treatment with 4,6-dinitro-*o*-cresol tends to abolish the acid production and to produce an increase in CO₂ retention.

Exactly comparable behavior of the eggs with respect to increased CO₂ partial pressure has been observed when the 4,6-dinitro-*o*-cresol was replaced by *p*-nitrophenol, 2,4-dinitrophenol, 2,4-dichlorophenol, or 2,4,5-trichlorophenol.

Fractionation of the Vitamin G Complex. BY SAMUEL LEPKOVSKY AND THOMAS H. JUKES. *From the Division of Poultry Husbandry, College of Agriculture, University of California, Berkeley and Davis*

Studies have been made of the "rat antidermatitis" vitamin (Factor 1), and of the filtrate factor, preventing a dermatitis in chicks (Factor 2). It was found possible to destroy Factor 2 in a

solution containing a mixture of Factors 1 and 2 by heating at pH 10 in a boiling water bath for 3 to 5 hours. The treatment left Factor 1 largely undestroyed.

Factor 2 is readily soluble in dry acetone, which may be used to extract Factor 2 from dry materials, such as skim milk powder, and from aqueous solutions. Some concentration of Factor 2 has been attained by removal of inert matter with alcohol, acetone, adsorbents, and inorganic precipitants. The basal diet used for the assay of Factor 2 was not affected by the addition of arginine.

A purified diet, containing the known vitamins in sufficient amount, including vitamins A, B (B₁), D, F, K, lactoflavin, the filtrate factor, the antiencephalomalacic factor of Goettsch and Pappenheimer, the gizzard factor of Almquist and Stokstad, and probably vitamin E, was fed to chicks. Growth was increased by the addition of 10 per cent of fat-extracted wheat germ to the diet.

The Basal Metabolic Rate of the Eskimo. BY VICTOR E. LEVINE.

From the Department of Biological Chemistry and Nutrition, Creighton University School of Medicine, Omaha

The basal metabolic rate of sixteen Eskimos and two whites living in the Arctic was studied during July and August, 1934. The natives in Akulurak, on the Yukon River 10 miles from the Bering Sea, subsist on salmon, white flour, sugar, and tea. Those in Nome, on the Bering Sea, instead of salmon consume seal meat and seal oil, which are used sparingly because of their scarcity.

Eleven Eskimos had a normal metabolic rate, while five had a rate ranging from +19 to +30. Heinbecker²¹ reporting on three Eskimos and Rabinowitch²² on ten Eskimos concluded that Eskimos have very high metabolic rates (+26 to +33) in comparison with people living in the temperate zone. These Eskimos lived largely on meat. Later Heinbecker reported normal rates for Eskimos on mixed diets.

Rabinowitch observed the common occurrence of polycythemia among Canadian Eskimos. We found anemia common among the Alaskan Eskimos of the Yukon-Bering Sea area. Increased basal metabolic rate has been reported in polycythemia. In anemia the basal metabolic rate may vary as a result of various

²¹ Heinbecker, P., *J. Biol. Chem.*, **80**, 461 (1928); **93**, 327 (1931).

²² Rabinowitch, I. M., *J. Nutrition*, **12**, 337 (1936).

factors, including anorexia, decreased muscular metabolism, and sedentary life. Seven out of twelve Eskimos whose basal metabolism was studied revealed a blood picture (hemoglobin, iron, and copper content) indicating marked anemia.

The basal metabolic rate of the Eskimo cannot be determined unless diet, season, bodily activity, blood picture, and physical condition are taken into account. The basal metabolic rate of the Eskimo does not bear a peculiar trait of race or climate.

Capillary Fragility Tests on Eskimos and Whites Living in the Arctic. BY VICTOR E. LEVINE. *From the Department of Biological Chemistry and Nutrition, Creighton University School of Medicine, Omaha*

Capillary fragility tests were made on 1046 people in the Arctic, 760 Eskimos, 5 men of Lapland descent, and 281 whites, living along the Bering Sea coast and the shores of the Arctic Ocean from St. Michael to Point Barrow inclusive. Capillary fragility was measured according to Dalldorf's method²² and expressed as the least negative pressure producing macroscopic petechiæ. Dalldorf's standards were adopted, the normal for groups on diets adequate in vitamin C being 35 cm. or more of mercury, and that for groups on diets poor in vitamin C being 25 cm. or less.

The capillary resistance of the 281 whites was normal in only 4 subjects; 21 had a resistance ranging between 30 cm. and 26 cm. Of the 765 natives, 70 had normal fragility, 48 had a range between 34 and 30 cm., and 100 between 29 and 26 cm. Subnormal capillary fragility was much more common in whites than in Arctic natives.

Ascorbic acid or tomato juice promptly raised capillary resistance. The prompt rise indicates that vitamin C is concerned in the physicochemical status of intercellular cement substance, not in its production. Breast-fed infants had higher capillary resistance than their mothers.

In a large series of tests capillary fragility seems to indicate the adequacy of vitamin C. The diets of whites and of Eskimos during 1935 (July to September inclusive) revealed marked deficiency in vitamin C. Eskimos and more especially whites in the Arctic very often live close to the border line of scurvy.

²² Dalldorf, G., *Am. J. Dis. Child.*, **46**, 794 (1933).

The Blood Iron and Blood Copper of Eskimos. BY VICTOR E. LEVINE, ADOLPH SACHS, AND A. APPELSIS FABIAN. *From the Department of Biological Chemistry and Nutrition, Creighton University School of Medicine, Omaha*

Blood iron and blood copper estimations were made on 62 Alaskan Eskimos, ranging in age from 10 to 50 years. The subjects were inhabitants of the Yukon-Bering Sea region living in Akulurak, St. Michael, Stebbins, King Island, and Nome.

The diet of the natives at the time, July 10 to August 15, 1934, was mixed, and consisted largely of salmon or seal meat and seal oil, white flour, sugar, and tea. Over 60 per cent had blood iron less than normal. As a rule hypoferronemia was accompanied by hypercupremia, a drop in blood iron resulting in an increase in blood copper.

The blood of the Eskimos that had a normal iron content gave a higher copper concentration than is generally found in the normal blood of white children or adults. The higher copper content may be due to the fact that marine animals have more copper than land animals.

Whipple and Robscheit-Robbins²⁴ in their classical experiments on anemia fed their dogs a basal ration with very low hemoglobin and red cell-regenerating power, consisting largely of white wheat flour and salmon baked into loaves. This ration very closely resembles that of the fish-eating Eskimos of the lower Yukon. Our results indicate that the fish-eating Eskimos have a greater tendency to anemia than the meat-eating Eskimos, the King Islanders and the Nome Eskimos.

Dielectric Constants of Aqueous Solutions of Certain Amino Acids and Related Substances. BY F. E. LINDQUIST AND CARL L. A. SCHMIDT. *From the Division of Biochemistry, University of California Medical School, Berkeley*

The present investigation was undertaken for the purpose of throwing further light on the question as to whether or not amino acids are micellated in aqueous solution.

The dielectric constants of aqueous solutions of glycine, alanine, proline, hydroxyproline, asparagine, and creatine were determined.

²⁴ Whipple, G. H., and Robscheit-Robbins, F. S., *Am. J. Physiol.*, **72**, 395, 419 (1925).

The method of measuring the dielectric constants was one of voltage resonance. A frequency of 3758 kilocycles was usually used.

The dielectric constants of aqueous solutions of glycine, alanine, and proline, after being corrected for the capacity of the cell to the surroundings, increased linearly with respect to the concentration, expressed as moles per liter, while those of hydroxyproline deviated slightly. The molal dielectric increment, δ , of glycine and alanine, when plotted against the temperature, yielded straight lines over the range of 1–30°, while that of hydroxyproline deviated somewhat.

Two types of dipolar association were assumed, and calculations were carried out to determine the effect of certain percentages of each type of association on the dielectric constants. The results indicate that if micellation of the three amino acids occurs, the amount is less than 5 per cent. The interpretation of the data is based on the assumption that a linear relationship between the dielectric constant and concentration of amino acid is evidence against the idea that the amino acid is micellated.

In the case of glycine, the conclusion that it is not appreciably associated in aqueous solution is not in agreement with the interpretation which has been placed on the freezing point data.

The Formation and Chemical Nature of the Bone Salt. BY MILAN A. LOGAN AND HENRY L. TAYLOR. *From the Biochemical Laboratory, Harvard Medical School, and the Forsyth Dental Infirmary, Boston*

The formation of the bone salts takes place in more than one step. The substance which forms first is tricalcium phosphate, and this adsorbs ions of salts, such as CaCO_3 , from the liquid phase. Hydrolysis and possibly solid solution also contribute to the composition of the precipitate finally obtained. The conclusion is based on the following experimental evidence.

1. Calcium phosphate was precipitated, at constant pH, in solutions containing bicarbonate. The precipitate first formed lost phosphate and added Ca^{++} and CO_3^{--} ions. The CO_2 taken up by the precipitate was proportional to the Ca^{++} concentration of the liquid.

2. The glycerol ash of bone and carbonate-containing calcium phosphate precipitates were partially dissolved by the continuous

addition of dilute acid at 100°. 60 to 80 per cent of the CO₂ was liberated, but only 5 to 10 per cent of the phosphate was dissolved. This indicates that the carbonate is on the surface of the crystals.

3. Varying amounts of bone and tricalcium phosphate were equilibrated with solutions of their ions. The results showed that the ion product $[Ca^{++}]^3 \times [PO_4^{=}]^2$ at equilibrium increased as the amount of solid equilibrated with a given amount of solution decreased. The results indicate that the precipitate adsorbs ions from solution. The solubility product obtained with minimum amounts approached pK_{sp} , 23.1, which indicates that the blood plasma is not supersaturated with respect to the bone salts.

The Effect of Hydrogen Ion Concentration on the Extractability of the Liver Proteins. BY JAMES MURRAY LUCK AND CHARLES COLVIN NIMMO. *From the Biochemical Laboratory, Stanford University, California*

Blood-free, frozen, powdered, dog liver, stored at -10° to -12° , was used as the source material and 5 per cent sodium chloride as the extracting medium. Below pH 3.5 the extractable salt-soluble protein was very small in quantity. At pH 4.5 to 5 the apparent salt-soluble protein content increased sharply. It tapered off above pH 7 but approached no clearly defined maximum.

The protein extracted at pH 7.2 was fractionally precipitated by adjusting aliquot portions of the solution to increasing degrees of acidity. The amounts remaining in solution were determined and plotted against pH. The solubility curve so obtained was identical up to pH 5 with that which resulted from similarly fractionating an extract made at pH 5.0. In the former case more protein could be extracted and present studies are directed toward determining whether this excess is the same, qualitatively, as that obtainable at pH 5.0.

Irrespective of whether the preliminary sodium chloride extractions were conducted at pH 5.0 or at 7.2, the subsequently isolated alkali-soluble fractions (globulin II) exhibited the same solubility-pH relationship; the two solubility curves were strictly superimposable above pH 6 and in close approximation below. globulin II was found to be almost completely insoluble at pH 5.3.

We conclude that globulin II is of a fixed composition and is independent in its constitution of the pH at which the preliminary salt extractions are made. As to whether globulin II exists preformed within the liver, or is a split-product which arises in the course of extraction with dilute sodium hydroxide, cannot yet be stated.

The Isolation of a New Compound from the Urine of Women with Adrenal Tumors. BY G. F. MARRIAN AND G. C. BUTLER.
From the Department of Biochemistry, University of Toronto, Toronto, Canada

A white crystalline substance, m.p. 243–244°, has been isolated from the neutral ether-soluble fraction of the urines collected from two women with adrenal tumors. It has not so far been isolated from any normal human urines which have been examined. This compound gave analytical figures corresponding to an empirical formula $C_{21}H_{36}O_3$. On treatment with acetic anhydride, it yielded a diacetate, m.p. 136.5°, which reacted with Grignard reagent with the evolution of gas, indicating that the 3rd oxygen atom was present as a non-reactive hydroxyl group, probably tertiary.

Iodine value determination by the method of Rosenmund and Kuhnhenh indicated that the substance was saturated. Oxidation with lead tetraacetate showed the presence of one glycol grouping. From the products of this oxidation were isolated acetaldehyde (as its 2,4-dinitrophenylhydrazone) and a ketone which yielded a semicarbazone, m.p. 264–265°.

The urines from the two patients were supplied by Mr. A. R. Broster of the Charing Cross Hospital, London. The authors are indebted to him for his whole hearted cooperation.

The Blood Precursor of Milk Fat. BY L. A. MAYNARD, ADRIAN HODSON, G. H. ELLIS, AND C. M. McCAY. *From the Laboratory of Animal Nutrition, Cornell University, Ithaca*

Initial studies of the free and total cholesterol in the inflowing and outgoing blood of the mammary gland of the cow by the Bloor colorimetric method suggested that the gland removes cholesterol ester fatty acids. This suggestion could not be confirmed by the digitonide method. A comparable study of the

two methods revealed that the higher values obtained with the colorimetric procedure were due to the presence in cow plasma extract of a chromogenic substance which was not cholesterol because saponifiable. Extensive studies of the free and combined cholesterol in arterial, jugular vein, and mammary vein plasma by the digitonide method revealed no changes indicating that cholesterol ester fatty acids are used to make milk fat.

By employing a modified procedure for isolating the phospholipids from plasma for their oxidative determination by the Bloor method, results have been obtained comparable to those obtained when the phospholipid is determined as lipid phosphorus. Studies of the inflowing and outgoing blood of the lactating gland by both methods were in agreement in furnishing no evidence that phospholipid fatty acids are removed by the gland.

A drop in total fatty acids was found in the blood passing through the gland, confirming earlier results. Calculating the triglyceride fatty acids as the difference between the total fatty acids and the sum of those present as cholesterol ester and as phospholipid produced data indicating that the neutral fat fraction of the blood is the precursor of milk fat.

Determination of Iodine in Ten Cc. of Blood by Burning in Platinum Combustion Tube with Screw Feed, and Distillation. BY J. F. McCLENDON, A. C. BRATTON, AND R. V. WHITE. *From the Laboratory of Physiological Chemistry, University of Minnesota Medical School, Minneapolis*

A Visking sausage casing (13 mm. \times 180 mm.) containing 10 cc. of blood is stretched in air by strings tied to each end while the blood is drying, and fed at the rate of 1.5 mm. per minute by means of a platinum-tipped screw feed into a platinum combustion tube. A series of five platinum spiral baffles insures complete oxidation of combustible gases. Three Fisher burners heat the tube to bright redness, and the products of combustion are passed through a two disk absorber containing 2 cc. of 5 per cent NaOH, 10 cc. of H_2O , and 1 cc. of 0.2 per cent Na_2SO_3 . The combustion proceeds in slightly diminished pressure (3 mm. of water), O_2 being supplied at the rate of 60 cc. per minute.

At the end of the combustion, the contents of the absorber are

combined with the ash in the tube and the tube washings (1 cc. of 0.5 per cent NaOH to 10 cc. of H_2O) and evaporated to 2 to 3 cc. in a 100 cc. all-glass still equipped with water condenser and air inlet tube (to prevent bumping). After acidification (3 cc. of 6 N H_2SO_4), 0.2 cc. of 4 per cent ferric chloride is added and the sample is evaporated nearly to dryness, the distillate being caught in 2 cc. of bromine water. The total distillate is then acidified with 0.5 cc. of 0.125 N H_2SO_4 and aerated to remove excess bromine. After cooling, 0.5 cc. of KI (25 mg. per cc.) is added and the liberated iodine titrated with 0.001 N $Na_2S_2O_3$, by a platinum-electrode differential method. The reference electrode is surrounded by liquid which has not been mixed with the last drop of $Na_2S_2O_3$.

The Growth of Algae in Synthetic Bromine-Free Medium. By A. R. MCINTYRE AND J. C. BURKE. *From the Department of Physiology and Pharmacology, University of Nebraska College of Medicine, Omaha*

In an effort to determine whether bromine is essential for growth processes a species of alga (*Pleurococcus vulgaris*) isolated in pure culture was grown upon the synthetic medium of Beijerinck, rendered bromine-free by successive saturation with chlorine and ebullition for 3 hour periods. Media thus prepared were subsequently shown to contain less than 1 part of bromine in 500 million by the fluorescein-cosin method of Hahn. Air was supplied to the culture flasks through a special wash tower containing 10 per cent $NaHCO_3$ for the removal of bromine and to allow the passage of CO_2 . By the above method of analysis samples of media supplied with air continuously for over 3 months were found to be bromine-free at the end of this period. In making the transfers to the growth flasks extremely small amounts of algae were used. Analysis showed that the flasks to which transfers had been made did not contain measurably greater amounts of bromine than blank flasks containing no transfers. Cultures grown under these conditions showed as rapid growth as control cultures grown in media treated identically but to which bromide was added. It is concluded that the rate of growth of

algæ (*Pleurococcus vulgaris*) in a synthetic, inorganic, bromine-free medium is no different from that of cultures in identical media but containing sodium bromide.

The Polysaccharides from Pig Gastric Mucosa. BY KARL MEYER, ELIZABETH M. SMYTH, AND JOHN W. PALMER. *From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York*

From the gastric mucosa of the pig, two separate and distinct polysaccharides were obtained. The first is neutral and sulfate-free, contains acetylglucosamine and galactose, and reacts in the serological test (performed by Dr. K. Landsteiner) as a blood Group A factor. It is apparently the substance responsible for the slimy nature of the gastric mucin. The second is an acid polysaccharide containing acetylglucosamine, hexuronic acid, and ester sulfate.

From the neutral polysaccharide glucosamine was isolated as the hydrochloride, and its concentration determined quantitatively. Galactose was identified by oxidation to mucic acid; it was also determined quantitatively. The ratio of the two sugars is apparently 1:1. From the acid fraction glucosamine was also isolated as the hydrochloride, and quantitatively determined. Hexuronic acid, acetyl, and acid-hydrolyzable sulfate were determined quantitatively. From the analytical data, the acid polysaccharide was proved to be a mucoitinsulfuric acid. Its concentration in the material is considerably lower than the neutral fraction. The specific rotation of the acid and neutral mucoitinsulfuric acid is identical with its isomeric chondroitin-sulfuric acid.

The acid fraction was separated from the neutral polysaccharide as the gelatin salt. Previous failures to recognize the neutral polysaccharide are probably due to its instability towards strong alkali.

A Spectrophotometric Method for the Determination of Methemoglobin in Hemoglobin Solutions. BY HARRY O. MICHEL. *From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina*

In the course of some work on the oxidation of hemoglobin to methemoglobin by various tissues,²⁵ the following procedure was developed.

The solution containing oxyhemoglobin and methemoglobin, which might be blood or a mixture of blood and various tissue proteins, was cleared with alumina gel or by half saturating with $(\text{NH}_4)_2\text{SO}_4$, diluted to a total pigment concentration of approximately 0.1 per cent, and KCN added to a concentration of 1 mM to convert methemoglobin to cyanhemoglobin without modifying the oxyhemoglobin. The ratio, R , of the extinction coefficients at 575 to 560 $m\mu$ is determined, and the per cent methemoglobin is then calculated from the equation:

$$\text{Per cent MHb} = \frac{90.6 - 52.4R}{0.457 + 0.067R}$$

The concentrations of HbO_2 and MHb can be calculated from the equations:

$$C_{\text{HbO}_2} = \frac{E_{560} \text{ (observed)}}{\frac{0.593 \% \text{ MHb}}{100 - \% \text{ MHb}} + 0.524} \quad C_{\text{MHb}} = C_{\text{HbO}_2} \frac{\% \text{ MHb}}{100 - \% \text{ MHb}}$$

The advantages of the method are its relative freedom from effects of pH and ionic strength, as contrasted with the commonly used alkaline methemoglobin method, and the large change in the ratio of the extinction coefficients with changing composition. The accuracy of the method is equal to that of the ordinary gasometric procedures.

A Comparison of the Hypervitaminoses Induced by Irradiated Ergosterol and Fish Liver Oil Concentrates. BY AGNES FAY MORGAN, LOUISE KIMMEL, AND NORA C. HAWKINS. *From the Laboratory of Household Science, University of California, Berkeley*

Nine series of experiments on rats were carried out in an attempt to discover whether moderately excessive doses of irradiated ergosterol and of fish liver oil concentrates produce toxic symptoms similar in kind and severity. Irradiated ergosterol and

²⁵ Bernheim, F., and Michel, H. O., *J. Biol. Chem.*, in press (1937).

concentrates of tuna liver oil and of cod liver oil along with a basal diet of constant composition were administered at levels of 4000 and 10,000 international units of vitamin D daily for periods of 21 to 57 days, after which serum calcium and inorganic phosphorus, bone ash, lung, heart, and kidney calcium and phosphorus content were determined. In some cases representative animals were sacrificed at 14 day intervals in order to follow the course of the changes induced.

In comparable animals lower percentages of femur ash and higher calcium and phosphorus contents of the soft tissues were usually found in those given the irradiated ergosterol than in those given the liver oil preparations. More deaths and greater interference with growth were observed also in the former group.

The effects of sex and of varying vitamin A intake upon hypervitaminosis D were studied also. No difference due to sex, apart from that ascribable to size, could be detected, but definite alleviation of symptoms was observed when large amounts of vitamin A, 2500 to 23,000 international units daily, were administered. No toxic effects of the large doses of vitamin A were seen.

The Influence of Kidney Excretion upon the Concentration of Serum Chloride and Base of the Dog during Exercise. By MINERVA MORSE AND FREDERIC W. SCHLUTZ. *From the Department of Pediatrics of the University of Chicago, Chicago*

The urine excretion of the dog during exercise on the treadmill has been studied in an attempt to account for the fact that during exercise the base to chloride ratio of the blood serum of the dog is reduced. This is not accounted for by the reversible shift of ions between serum and cells.

The dog was kept in a metabolism cage; 24 hour samples of urine were collected for 3 days preceding and 2 days following exercise. The dog ran an average of 5 hours. Water was not allowed during the exercise period, but *ad libitum* at all other times.

The following observations were made: (1) The volume output of urine for the 24 hours including and following the exercise period increased to almost twice the average daily output preceding exercise. (2) In spite of the increased volume, a slight decrease in excretion of total fixed base and a marked decrease in that of

chloride occurred. (3) There was little change in phosphate excretion, but an increase in excretion of bicarbonate and sulfate.

Such a selective excretion by the kidney during exercise, whereby the normal proportion of chloride to base in the urine is reduced, would serve to explain, in large part, the decrease in the base to chloride ratio of the serum which occurs during exercise.

The Effect of Changes in Diet on the Volume and Composition of Rat Milk. II. BY ARTHUR J. MUELLER AND WARREN M. COX, JR. *From the Research Laboratory, Mead Johnson and Company, Evansville, Indiana*

Mother rats were placed on purified diets at parturition, and milked on the 18th and 20th days of lactation. A minimum of five rats was used in each group, and the milk obtained from each studied separately. Three dietary ingredients have been varied: protein, yeast, and calcium and phosphorus.

Stock Rats—The variation in protein, fat, calcium, phosphorus, magnesium, and ash from individual samples of stock rat milk has been determined.

Protein: Levels of 5, 10, 20, 30, and 50 per cent purified casein were fed. The milk yield and the weight of sucklings increase with the protein level. The percentage protein in the milk is greater at a 50 per cent dietary level. At a 5 per cent level, fat and ash content were high, and protein content low. *Yeast:* Decrease in the level of yeast (protein constant at 10 per cent) from 8 to 0 per cent, progressively, resulted in decreased milk volume, smaller young, and progressively greater weight loss by the mother rats. The percentage of fat and protein in the milk was increased only with zero yeast; per cent ash was unaffected. *Calcium and phosphorus:* Ratios of Ca/P greater than 1.0 (constant P) resulted in progressively smaller young and decreased ash, calcium, and phosphorus content of the milk. Ratios greater than 2.0 resulted in rapid decline of milk production. Ratios of Ca/P less than 1.0 (constant Ca) had no significant effect on the volume of milk, weight of young, or percentage calcium, phosphorus, or ash in the milk. The number of young raised, however, was reduced.

Effect of Lactoflavin and Vitamin B₆ Deficiency on Tissue Metabolism. BY JYTTE MUUS, OTTO A. BESSEY, AND A. BAIRD

HASTINGS. *From the Department of Biological Chemistry, Harvard Medical School and Harvard Dental School, Boston*

The inhibition of growth produced, when either the flavin factor or the B₆ factor of the vitamin B complex is omitted from the diet of young rats, led to the investigation of the metabolism of the excised tissues of such animals. After 8 weeks on the experimental diets, the oxygen consumption of the diaphragm and liver was measured by the Warburg technique on the following groups of animals: (1) twelve normal controls on a balanced stock diet; (2) eleven controls on the experimental diet with all vitamin B complex factors added; (3) eleven rats on a diet deficient in the flavin factor; (4) seven rats on a diet deficient in the vitamin B₆ factor; (5) seven rats deficient in both the flavin and vitamin B₆ factors.

Absence of the flavin factor did not influence the metabolism of the liver, but that of the diaphragm was 30 per cent above normal. On histological examination, no abnormality of the muscle was observed except greater concentration of nuclei.

Absence of the vitamin B₆ factor resulted in a decrease in the metabolism of the liver amounting to 24 per cent, whereas the metabolism of the diaphragm was normal. Histological examination revealed fatty infiltration of the liver but no abnormality of the muscle, save that of greater concentration of nuclei.

When both the flavin and vitamin B₆ factors were absent, the metabolism of the liver was decreased 24 per cent and the metabolism of the diaphragm was increased 38 per cent. The clinical and histological changes of the same degree of severity appeared earlier in this group than when either factor alone was absent.

Vitamin D Potency of Irradiated Milk As a Function of Energy Input. BY BRIAN O'BRIEN, H. DOUGLAS McEWEN, AND KENNETH MORGAREIDGE. *From the Institute of Optics and the Department of Biochemistry, The University of Rochester, Rochester, New York*

Whole milk has been irradiated in a bubble-film irradiator, with a carbon arc under controlled conditions. Vitamin D potency has been determined by a standardized technique²⁶ which includes comparison with the international standard in every assay.

²⁶ O'Brien, B., and Morgareidge, K., *Proc. Soc. Exp. Biol. and Med.*, **32**, 118 (1934).

The curve of potency against energy input has been found to fit, approximately, the expression, $P = P_{\max} (1 - e^{-aE})$, up to energy inputs to the arc of approximately 100 joules per gm. of milk irradiated. Above this input, potency falls below values predicted by the equation and, for most milks, reaches a maximum of about 1.6 international units per gm. (3.5 per cent fat content) at an input of 300 joules. Exceptional milks of the same fat content may develop double this potency but the majority of samples taken at random from different localities at different seasons agrees closely with this average.

Energy inputs up to 2100 joules per gm. have been used. The potency decreases progressively for inputs in excess of 300 joules per gm. At the highest input level, the potency of an average sample, determined by feeding from 2 to 10 days following irradiation, was 1.1 international units per gm. The same sample, following storage at 2° for 20 additional days, had a potency of 0.4 international unit per gm. Except in flavor, no gross changes in the milk were detectable. The same milk, irradiated at 150 joules per gm., showed no decrease in potency on similar storage. For energy inputs intermediate between 300 and 2100 joules, loss of potency on storage increased progressively with input.

Paralysis in the Young of Vitamin E-Deficient Female Rats.

By H. S. OLCOTT. *From the Biochemical Laboratory, State University of Iowa, Iowa City*

The paralysis in the young of vitamin E-deficient female rats is accompanied by and probably due to a muscular dystrophy²⁷ similar in character to that observed in Herbivora on deficient diets (Goettsch and Pappenheimer, and others). Preliminary observations indicate that the dystrophy is present in milder form in animals which show no external symptoms of paralysis or weakness, and that the administration of vitamin E prior to the expected time of onset alleviates the severity of the dystrophy.

Rate of Citric Acid Formation Following the Injection of the Sodium Salts of Certain Dicarboxylic Acids. BY JAMES M.

ORTEN AND ARTHUR H. SMITH. *From the Laboratory of Physio-*

²⁷ I am indebted to Professor H. P. Smith of the Department of Pathology for his help in the interpretation of the sections.

logical Chemistry, Yale University School of Medicine, New Haven

Amounts of the disodium salts of certain dicarboxylic acids supplying 100 mg. of sodium per kilo of body weight were injected intravenously into four dogs fed a citrate-low ration. The quantity of citric acid excreted in the urine was determined for a 1 hour period immediately preceding the injection and then at definite intervals thereafter. Equivalent amounts of sodium as the chloride and bicarbonate were administered as control substances.

Only slight increases in citric acid excretion occurred following the injection of the chloride or bicarbonate, whereas marked rises were observed after the administration of malate, fumarate, succinate, or malonate. The citric acid output increased sharply during the first half hour after injection of the dicarboxylic acid salts and rose to a maximum in 1 hour; a progressive, rapid decrease then occurred, the value receding to the original basal level within $4\frac{1}{2}$ hours after the injection.

Simultaneous analyses of the citric acid content of the blood and urine were made following the injection of sodium malate. Samples were taken before the injection and at definite intervals thereafter. In contrast to the marked rise in the citric acid content of the urine after the administration of malate, only slight increases in blood citric acid were observed. The values obtained at 15 and 30 minute intervals after injections were slightly but consistently higher than the basal level; however, within an hour the values were almost identical with those observed prior to the injection.

Biological Assay of Vitamin E; Application to Wheat Germ and Wheat Germ Oil.* BY LEROY S. PALMER. *From the Division of Agricultural Biochemistry, University of Minnesota, St. Paul*

The vitamin E unit of Evans and associates²² has a variable quality in terms of dosage, percentage of live litters, or number of young born (living or living and dead). In this study the Evans

* The products tested in this investigation were furnished through the courtesy of General Mills, Inc., Research Laboratories, Minneapolis.

²² Evans, H. M., Murphy, E. A., Archibald, R. C., and Cornish, R. E., *J. Biol. Chem.*, **108**, 515 (1935). Evans, H. M., Emerson, O. H., and Emerson, G. A., *J. Biol. Chem.*, **112**, 319 (1936).

and Burr²⁹ single oral dose procedure and other uniform conditions were applied and the live litter efficiency (percentage of pregnant females which bore living young) compared with the placental implant efficiency (percentage of placental implants which resulted in the birth of living young). In nearly 50 tests involving three to six rats each, the correlation between live litter efficiency and placental implant efficiency was $r = +0.69$, a significant value. Twenty tests involved ten products tested at two levels. Correlations between the relative levels and the corresponding relative efficiencies were insignificant for both types of efficiency. Apparently the assay of vitamin E is at best merely a qualitative assay and does not yet warrant interpretation in terms of units. A reference standard, if available, probably would not make results now obtainable more quantitative.

The vitamin E assay procedure applied to various vitamin E-containing products could be interpreted, qualitatively, to show (1) a close correlation between the vitamin E in raw or processed (Embo) wheat germ, on the oil content basis, and the pure oil expressed from the fresh germ; (2) a high retention of the vitamin in the processed wheat germ (Embo) kept for 1 year at room temperature in a sealed, evacuated can; (3) a high stability of vitamin E in the expressed oil kept for several months at refrigeration temperatures in sealed containers, either glass or tin.

Experimental Alcaptonuria in the White Rat. BY EVANGELINE PAPAGEORGE AND HOWARD B. LEWIS. *From the Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor*

The production of experimental alcaptonuria has an important bearing on the question of the status of homogentisic acid as a possible intermediary product in normal aromatic amino acid catabolism. By feeding diets containing large amounts of *l*-phenylalanine to white rats over a considerable period of time so that the daily intake of amino acid exceeded 0.3 gm. per 100 gm. of body weight, we were able repeatedly to demonstrate an alcaptonuria which ceased when the basal diet containing no phenylalanine was resumed. The urine of the alcaptonuric rats gave the qualitative tests characteristic of the urine of clinical alcaptonuria

²⁹ Evans, H. M., and Burr, G. O., *Mem. Univ. Calif.*, 8 (1927).

and showed an increase in the daily total "apparent phenol" content to a value several times greater than the average value of urines of control rats. Conclusive evidence of the excretion of homogentisic acid was obtained by the preparation from the urine of the alcaptonuric animals of pure dibenzoylhomogentisamide, the identity of which was established by analyses.

This is, to our knowledge, the first report of the successful production of a true experimental alcaptonuria with the exception of Abderhalden's single experiment with tyrosine in man, which he was unable to repeat.

The Storage in the Body Organs of the Factor Protective against the Injury Due to Dietary Egg White. BY HELEN T. PARSONS, JANE G. LEASE, AND DORIS JOHNSON. *From the Department of Home Economics, University of Wisconsin, Madison*

The concentration of the potent factor in the body organs of rats and chicks can be varied by feeding different amounts of the factor with diets rich in egg white. The concentration varies also according to the species of animals. A commercial sample of turkey liver is the richest source thus far tested. Fifteen samples of human liver showed wide variation in concentration.

The Effect of Acid Hydrolysis on the Yield of Androgenic Activity from Human Urine. BY D. H. PETERSON, T. F. GALLAGHER, AND F. C. KOCH. *From the Department of Biochemistry of the University of Chicago, Chicago*

Previous comparative studies on the yield of androgenic activity from unacidified urine and urine boiled for 2 hours with 10 per cent by volume of hydrochloric acid gave no definite evidence of the presence of conjugated forms of androgens in human urine. However, boiling for 15 to 30 minutes with 10 per cent by volume of hydrochloric acid increases the yield of androgenic activity 70 to 160 per cent over the no boiling procedure and 35 to 78 per cent over the 2 hours boiling procedure. Obviously a non-extractable form is liberated by the brief acid hydrolysis and then some form of androgen is destroyed by more prolonged acid hydrolysis. Atmospheric oxygen is not a factor in this destructive process and control studies indicate that androsterone is not destroyed by acid hydrolysis. Possibly dehydroandrosterone is converted into the

inactive chlorodehydroandrosterone or possibly a dehydration of an androgen by hydrochloric acid into an inactive form is responsible for the loss of activity. Urine from women as well as men exhibits this behavior.

The Determination of the Rate of Emptying of the Rat Stomach Following Intra-gastric Administration of Glucose Solutions.

BY H. B. PIERCE, LORRAINE HAEGE, AND PAUL F. FROESCHLE.
From the Department of Vital Economics, The University of Rochester, Rochester, New York

The object of this investigation was to determine the emptying time of the stomach after the intra-gastric administration of glucose solutions to adult rats fasted for 24 hours and anesthetized with pentobarbital. The dilution of the sugar solution in the stomach was determined during the course of the experiment with the hope that information concerning one of the probable factors involved in the absorption of glucose from the small intestine could be obtained.

An incision was made through the abdominal wall of the animal, starting at the xiphoid process of the sternum and continuing about an inch caudad. The duodenum was then traced and a glass cannula inserted with its tip about half an inch from the pylorus. Sugar was fed in solution by stomach tube, and the stomach contents passing into the cannula were removed from time to time. The volume of the sample, the time of removal, and the sugar content were noted.

It was found that there was a decrease in the volume and sugar content of the fluid as time progressed. The concentrated sugar solutions were diluted about 300 per cent within an hour after feeding, the dilution rate decreasing with time. As with humans, there seems to be considerable variation in the emptying rate of the stomach.

A Volumetric Method for the Determination of Acacia in Serum, Lymph, and Urine. BY MARSCELLE H. POWER. *From the Division of Biochemistry, The Mayo Foundation, Rochester, Minnesota*
The principle of the method has been referred to previously.²⁰

²⁰ Power, M. H., Keith, N. M., and Wakefield, E. G., *Am. J. Physiol.*, **113**, 107 (1935).

In narrow tipped centrifuge tubes trichloroacetic acid filtrates of serum and lymph or dilutions of urine are mixed with acetone in the proportion of 2 cc. of filtrate to 5 cc. of acetone. After the tubes have stood an hour or more, the resulting precipitates of acacia are separated in the centrifuge, washed with acetone, and dried. The precipitates are oxidized quantitatively by heating 10 minutes in boiling water with $K_2Cr_2O_7$ (1 cc. of 0.5 to 1.0 N) and concentrated H_2SO_4 (2 cc.), after which the mixtures are cooled, diluted, transferred to Erlenmeyer flasks, and the residual $K_2Cr_2O_7$ determined iodometrically.

Precipitation of acacia from known solutions is substantially complete; *e.g.*, the dichromate required to oxidize directly 0.25 to 2.0 mg. of "purified" acacia ranged from 2.76 to 2.80 cc. of 0.05 N per mg., whereas the precipitates obtained when the same quantities in solution in 5 to 10 per cent trichloroacetic acid were treated with acetone required 2.70 to 2.74 cc. of 0.05 N per mg. Filtrates of acacia-free human serum contain substances which precipitate with acetone equivalent to approximately 0.05 cc. of 0.05 N dichromate per cc. of serum. When a correction of this order is applied, the recovery of known amounts of acacia added to serum is satisfactory. Alcohol is somewhat inferior to acetone as a precipitant for acacia.

A Chemical Reagent for the Detection and Estimation of Vitamin B₁. BY H. J. PREBLUDA AND E. V. MCCOLLUM. *From the Biochemical Laboratory, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore*

We have found that derivatives of aniline or the naphthylamines, after being treated with nitrous acid, produce characteristic colored solutions when allowed to react under certain conditions with vitamin B₁. Either *p*-aminoacetanilide or *p*-aminoacetophenone is suitable for this purpose. Under proper conditions these reagents react with the vitamin to form purple-red compounds which crystallize readily, are stable, and are highly insoluble in the aqueous medium in which they are formed.

The respective reagents produced from these two amines have been successfully used for obtaining the corresponding crystalline derivatives from Merck's crystalline vitamin (natural and synthetic), from wheat germ, rice polishings, Seidell's international

adsorbate, Lilly's adsorbate, Anheuser-Busch's yeast concentrate, and the synthetic vitamin (betaxin) of the Winthrop Chemical Company. In each case the characteristic colored crystalline compound was obtained. The crystalline colored product obtained by the use of either reagent is readily extractable by suitable selective solvents, such as acetone or isobutyl alcohol.

Since the color of the reaction product with these reagents is sufficiently sensitive to enable one to detect the presence of the respective compounds in amounts corresponding to one or more international units in 1 cc. of solution, the test is now being made the basis for an accurate assay (a quantitative assay) of the vitamin in biological materials and foodstuffs.

Observations on Anaerobic Glycolysis in Brain. By J. H. QUASTEL AND A. H. M. WHEATLEY. *From the Biochemical Laboratory, Cardiff City Mental Hospital, Cardiff, Wales*

If brain cortex slices are deprived of glucose and exposed to nitrogen for a few minutes, the tissue loses most of its power of anaerobic glycolysis. This is not due to the breakdown of the tissue cells or enzymes under anaerobic conditions in the absence of glucose nor to loss of a coenzyme by diffusion, for the phenomenon is quite reversible. This may be shown by exposing brain cortex slices, made incapable of anaerobic glycolysis by previous exposure to nitrogen in the absence of glucose, to oxygen, adding glucose, and exposing again to nitrogen. The brain slices are now found to have regained all their previous power of breaking down glucose anaerobically.

Anaerobic glycolysis of brain is greatly dependent on the calcium ion concentration of the medium in which the tissue slices are immersed. Anaerobic glycolysis of brain in the absence of added Ca^{++} may be increased over 100 per cent by 0.001 M Ca. Potassium ions depress anaerobic glycolysis. Thus it may be stated that while K ions increase and Ca ions depress respiration of brain tissue, the effects of these ions are reversed in anaerobic glycolysis. In the absence of Ca^{++} , both Sr^{++} and Mg^{++} will increase anaerobic glycolysis, the effect of strontium being of the same order as that of Ca, but that of Mg being decidedly less. All these ions show optimum concentrations in their accelerating effects on anaerobic glycolysis.

Anaerobic glycolysis by brain cortex slices is also greatly accelerated by the presence of nicotine, pyridine, and pyrrole.

Quantitative Determination of Prothrombin. BY ARMAND J. QUICK AND MADELINE LEU. *From the Department of Pharmacology, Marquette University School of Medicine, Milwaukee*

The method is based on the assumptions that (1) the rate of coagulation is a function of the concentration of thrombin; (2) the production of thrombin in oxalated plasma is proportional to the concentration of prothrombin if an excess of thromboplastin is present and an optimum amount of calcium is added. The test is as follows: 9 cc. of blood are mixed with 1 cc. of 0.1 M sodium oxalate and centrifuged. In a small test-tube immersed in a water bath kept at 40°, 0.1 cc. of plasma is mixed with 0.1 cc. of a thromboplastin emulsion prepared from rabbit brain, 0.1 cc. of 0.025 M calcium chloride is quickly added, and the clotting time noted. By mixing normal plasma with plasma from which the prothrombin had been removed with aluminum hydroxide, solutions with varying concentrations of prothrombin were prepared. Their clotting times were determined and from these values a curve was constructed which was found to correspond to the equation, $t = a + (k/c)$ (a and k are constants, c the concentration of prothrombin, t clotting time). For rabbit blood, $a = 5.13$, $k = 139.4$; while for human blood, $a = 10.29$, $k = 353.72$. The value of the constants varies somewhat with the potency of the thromboplastin employed. The ones given were obtained with a highly active preparation. Human blood was found to contain only one-fifth as much prothrombin as rabbit blood.

The Relation of the Antithrombogens, Heparin and Calcomine-Fast Pink, to the Antithrombin Normally Occurring in Blood.

BY ARMAND J. QUICK. *From the Department of Pharmacology, Marquette University School of Medicine, Milwaukee*

Previously it has been shown that (1) plasma shows no antithrombic action when tested with thrombin; nevertheless, after the coagulation of the fibrinogen, it will neutralize large amounts of thrombin; (2) heparin, which itself is not an antithrombin, since it does not inhibit the coagulation of purified fibrinogen by thrombin, will form a strong antithrombin when added to plasma.

It has now been found that the antithrombic action of serum resides in the albumin fraction; furthermore, that heparin is antithrombic only in the presence of serum albumin. This suggests that serum albumin possesses the power to bind and inactivate thrombin, but that it manifests this action only after all the fibrinogen has been clotted; the latter, being the normal substrate, apparently has a greater affinity for thrombin than albumin. The addition of heparin to serum albumin presumably so increases the affinity for thrombin that it exceeds that of fibrinogen, and consequently combines with thrombin before coagulation can occur. Apparently, the active antithrombic agent is a heparin-albumin complex. The dye, calcomine-fast pink, also is antithrombic in plasma, but it was found that its action is on fibrinogen. Thus, in a system in which the amount of thrombin and the dye is kept constant, the coagulation time increases as the fibrinogen is decreased. Furthermore, when calcomine-fast pink is added to purified fibrinogen, a precipitate is formed. This does not occur in plasma, probably because of the protective colloidal action of the other serum proteins. Germanin appears to act like calcomine-fast pink.

The Response of Goitrous Rats to Iodide Administration. BY

ROE E. REMINGTON AND ARTHUR M. LASSEK. *From the Medical College of South Carolina, Charleston*

Following the determination of the minimal protective dose of iodine in the rat (1 to 2 micrograms per day), it seemed desirable to determine the curative effect of iodide on goitrous rats. Young rats, born of goitrous parents, were reared to 9 weeks of age on the iodine-deficient diet of Remington,¹ when a representative group had thyroids weighing 2.7 times normal, with 22.1 per cent dry matter and 0.007 per cent iodine (dry basis), compared with normals of 33 per cent and 0.20 per cent. The same diet for 6 weeks longer did not further increase the hyperplasia.

A group given 5 micrograms per day of iodine as iodide in the diet for 6 weeks yielded glands which had regressed to 1.35 times normal, dry matter 28.75 per cent, iodine 0.195 per cent. Histologically they appeared normal or nearly so. It is questioned

¹ Remington, R. E., *J. Nutrition*, 13, 223 (1937).

whether iodides can cause full return to normal weight and composition.

A third group received four doses at intervals of 2 weeks, of a solution containing 10 micrograms of iodine as iodide, in the hope that periodic stimulation by inadequate doses might increase the hyperplasia. The degree of enlargement was 2.2, dry matter 24.69 per cent, indicating slight regression. Histologically this degree of recovery could not be readily differentiated from the most goitrous group.

The Biological Synthesis of Cholesterol As Studied with the Use of Deuterium. BY D. RITTENBERG. *From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

The body fluids of a group of mice were maintained at a concentration of 1.5 atoms per cent deuterium over a period of time. The deuterium content of the cholesterol from these mice was determined at intervals. As in the case of the fatty acids, synthesis of the cholesterol is accompanied by the introduction of stably bound deuterium. After 60 days the deuterium to protium ratio was one-half that of the body fluids. Thus, at least 22 of the hydrogen atoms of the cholesterol molecule were exchangeable with the body fluids at some stage of its synthesis. These facts indicate that cholesterol is synthesized in the animal from numerous smaller units.

The Estimation of Albumin and Globulin in Blood Serum. I. A Study of the Errors Involved in the Filtration Procedure. BY HOWARD W. ROBINSON, J. WAIDE PRICE, AND CORINNE G. HOGDEN. *From the Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati*

Howe's protein partition method (22 per cent Na_2SO_4) requires a filtering medium to remove globulin. In his first paper, Howe states, "In using the procedure described, it is assumed that the aliquot taken from the filtrate after precipitation contains a true proportionate amount of the unprecipitated protein and that there has not been any adsorption of the unprecipitated protein by the precipitated protein nor by the filter paper." After using

the method with various types and quantities of filter paper, we believe that this adsorption factor discussed by Howe cannot be ignored.

Experiments show that the amount of protein in filtrate is dependent on type, size, and quantity of filter paper and on amount of solution filtered. From concentrated salt-protein solutions, even in the absence of precipitated globulin, filter paper removes slowly a definite amount of protein. Thus, the common practise of refiltering many times to obtain a clear filtrate has a tendency to lower the albumin concentration. With the most satisfactory paper thus far tested (one sheet, Whatman No. 50), this value is approximately 0.25 gm. per cent, which in some instances makes a great difference in the calculated albumin to globulin ratio. The amount of solution filtered is also an important factor, a difference being obtained between 15.5 and 31 cc. of the protein-salt solution. By filtering larger amounts of solution (45 cc. plus) an ultimate constant and higher albumin value are obtained, independent of the paper employed. Our data indicate that comparable results may be obtained only when the procedure is followed exactly in all details.

Further Studies of the Metabolism of Heptoses. BY JOSEPH H. ROE. *From the Department of Biochemistry, School of Medicine, George Washington University, Washington*

Previous work²² has shown that *d*-mannoheptulose (*d*-manno-ketoheptose) is physiologically available to the rabbit. A similarly executed study of *d*-mannoheptose indicates that this sugar is not utilized by the rabbit. Further studies of *d*-mannoheptulose are in progress.

Partition and Determination of Blood Lipids. BY ANTON R. ROSE, FRED SCHATTFNER, AND WILLIAM G. EXTON. *From the Laboratory and Longevity Service of The Prudential Insurance Company of America, Newark*

The lipids in a single (1 cc.) sample of blood are partitioned by their known solubilities. The system is designed for clinical work and takes less time than other procedures.

²² Roe, J. H., and Hudson, C. S., *J. Biol. Chem.*, **112**, 443 (1935-36).

In the first step the blood is deproteinized with acetone, anhydrous sodium sulfate being used as a diluent and dehydrant. Magnesium chloride is also added to increase the solubility of the fatty acid soaps and decrease the solubility of lecithin.

The phospholipids are recovered from the powdery protein-sodium sulfate mass with ether and saponified. An aliquot of the acetone liquid is taken for the cholesterol ester and the remainder is made alkaline with alcoholic sodium hydroxide. If a precipitate forms, the fatty acids are measured as a soap and credited to the non-ester fatty acids of the blood. The acetone is evaporated and cholesterol extracted from its residue with petroleum ether. The total cholesterol and ester fraction are then determined by the Liebermann-Burchard color reaction. The soaps are digested in cold, dilute sulfuric acid to liberate the fatty acids, which are taken up in 1 cc. of alcohol and treated with calcium gluconate to obtain a reproducible milky suspension. The cholesterol is measured colorimetrically. All of the other fractions are measured turbidimetrically. The Universal electroscometer, which gives better precision than has hitherto been possible with nephelometers, is used for both measurements.

Bone Growth in Normal and Rachitic Rats. BY WALTER C. RUSSELL, M. W. TAYLOR, AND MARION T. DUNCAN. *From the Department of Agricultural Biochemistry, New Jersey Agricultural Experiment Station and Rutgers University, New Brunswick*

Radii have been obtained from albino rats on a stock colony ration at 3 day intervals for the age period of 3 days to 54 days, inclusive. From 24 to 54 days of age, inclusive, litter mates of the stock colony group were fed the Steenbock²² rachitogenic Ration 2965 and the radii taken at 3 day intervals.

The split, stained radii were photographed and the area and the average width of the epiphyseal cartilage measured. These values for the normal, or stock colony, animals decreased continuously and became practically constant at 48 days of age. When animals were placed on the rachitogenic ration, the narrowing of the epiphyseal cartilage ceased within 3 days and a widen-

²² Steenbock, H., and Black, A., *J. Biol. Chem.*, **64**, 274 (1925).

ing process began which continued until the animals had been on the rachitogenic ration for 18 days (42 days of age), after which there was no further widening of the cartilage.

The progress of line formation in rachitic rats fed an antirachitic substance during the first 3 days of a 12 day period was followed by measuring the areas of new calcification at 4, 6, 8, 10, and 12 days. Although complete agreement of results was not obtained, line formation usually reached a maximum in 8 to 10 days.

Studies on the Benedict-Denis Procedure for the Determination of Total Sulfur in Biological Materials. BY CHARLES B. RUTENBER AND JAMES C. ANDREWS. *From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*

Recent criticisms²⁴ of the Benedict-Denis procedure for total sulfur, when applied to methionine, prompted a detailed study of this method. Although our results with both cystine and methionine were higher than those of Painter and Franke, we confirm their conclusion that methionine sulfur is incompletely oxidized.

Use of the original Denis reagent on pure methionine produced recoveries of less than 80 per cent of the theory. Addition of 11.25 milli-equivalents of Na_2CO_3 to 10 cc. of the reagent raises the recovery to about 97 per cent. Addition of larger amounts of Na_2CO_3 produces a rapid decrease in sulfur recovery with much more erratic results. The optimum region of the curve of recovery *versus* alkalinity is very narrow: 10 to 12 milli-equivalents of Na_2CO_3 per 10 cc. of Benedict-Denis reagent. As usual, theoretical recoveries were obtained from pure cystine regardless of the amount of alkali added.

The prolonged ignition often recommended is objectionable if a free gas flame is used, because of variable amounts of SO_2 in the gas. Blank tests on the reagent and on Na_2CO_3 showed that prolonged ignition (2 hours) can produce nearly 50 mg. of BaSO_4 , particularly when the dish is tilted and the contents exposed to the free flame. Heating in an electric muffle is therefore preferable.

²⁴ Painter, E. P., and Franke, K. W., *J. Biol. Chem.*, **114**, 235 (1936).

The Secretion of Sodium Cholate into the Bile As Affected by Thyroxine. BY L. H. SCHMIDT. *From the Christ Hospital Research Institute, and the Department of Biochemistry, University of Cincinnati, Cincinnati*

Previous experiments have shown that sodium cholate, injected intravenously, disappears rapidly from the blood of normal rabbits, but is retained for a much longer time by rabbits treated with thyroxine. The present results show that this retention is due primarily to an alteration in the capacity of the liver to secrete sodium cholate into the bile.

The biliary secretion of sodium cholate was studied in four groups of rabbits with biliary fistulæ: (1) normal controls, (2) normals, injected with sodium cholate, (3) thyroxine-treated controls, and (4) thyroxine-treated animals, injected with sodium cholate.

Control rabbits, treated with thyroxine, secreted a smaller volume of bile than normal controls, but thyroxine treatment had no effect on either cholic acid or total conjugated bile acid concentrations.

After the injection of sodium cholate, the bile volume increased in both normal and thyroxine-treated rabbits; however, this increase was much greater in the normal animals. The cholic acid concentration also increased to a greater extent in the normal group; in these animals the maximum concentration of cholic acid was secreted 20 minutes after sodium cholate injection, as compared with 65 minutes for thyroxine-treated animals.

Consequently, the injected cholic acid was recovered more rapidly in the bile of the normal animals; at the end of 65 minutes recovery amounted to 75 per cent in the normals and 45 per cent in the treated rabbits.

Studies in the Intermediate Metabolism of the Fatty Acids with Deuterium As an Indicator. BY RUDOLF SCHOENHEIMER. *From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*

In the study of fatty acid metabolism with the aid of deuterium, two general methods are available. First, deuterio-fatty acids are given to the animal and the deuterium content of other body constituents and the body fluids determined. Secondly, the

synthesis of the acid in the animal is studied by maintaining the body fluids at a deuterium concentration higher than normal. By combining these methods it was found that butyric acid when given to mice is rapidly burned. None of the butyric acid is stored, either as such or after conversion into other fatty acids. Caproic acid is handled in a similar manner to butyric acid. The higher fatty acids, however, are deposited to a large extent in the tissues after ingestion and undergo interconversion; that is, stearic acid is not only desaturated to oleic acid but is also degraded to palmitic acid.

The Thermostability of Some of the Components of the Vitamin B Complex in Yeast. BY HAROLD W. SCHULTZ. *From the Biochemical Laboratory, State University of Iowa, Iowa City*

Dried yeast autoclaved for 6 hours and heated dry at 120° for 36 hours produced growth in rats receiving a vitamin B-free diet plus crystalline vitamin B₁. The growth rate of rats fed only small amounts of the heated yeast was not increased by a concentrate of the filtrate factor or by an alcohol-ether-insoluble fraction prepared from yeast extract after treatment with fullers' earth at pH 1. The fullers' earth adsorbate, on the other hand, did increase the growth rate.

A fullers' earth adsorbate (at pH 1) prepared from an extract of heated yeast together with the filtrate and vitamin B₁ permitted growth. A flavin concentrate from whey powder (Booher) together with the same filtrate and vitamin B₁ did not, thus indicating the presence of another component, besides flavin, in this adsorbate.

Concentrates of Factor 1 and Factor 2 (Lepkovsky) were prepared from a yeast extract (adsorption at pH 4.8). When these concentrates were fed with crystalline vitamin B₁ and crystalline flavin, rats grew without developing any type of lesions. Heated yeast (0.5 gm. daily) replaced Factor 1 or Factor 2 equally well, but did not completely replace flavin. The constituent in Factor 1 is probably vitamin B₆, since it prevents dermatitis. It cannot yet be stated whether the component in Factor 2 is the filtrate factor or the precipitate factor. Both may be present.

The heating of yeast, as described, does not so completely inactivate some of the newer components of the vitamin B complex as has been supposed. Flavin is apparently the most vulnerable.

Determination of Carbohydrates in Proteins. BY FAY SHEPPARD AND MARK R. EVERETT. *From the Department of Biochemistry, University of Oklahoma Medical School, Oklahoma City*

The conjugation product of furfural and tryptophane (humin) in sulfuric acid solution lends itself to the quantitative colorimetric determination of carbohydrates, other than glucosamine, by the following procedure.

To 1 cc. of protein solution (containing 0.05 to 0.5 mg. of carbohydrate), in a 50 cc. Erlenmeyer flask, add 1 cc. of 1 per cent tryptophane (in 1 per cent sodium benzoate solution) and 7 cc. of 77 per cent sulfuric acid (by volume). Mix and heat 20 minutes on a boiling water bath. Cool 5 minutes in an ice bath in the dark. Read in the Pulfrich photometer, using filter No. S-53 (or No. S-50) with appropriate compensating blanks, and refer to curves constructed from pure sugars.

Quantitative measurements of glucose, mannose, galactose, glucuronic lactone, and glucosamine, alone and added to protein, demonstrate certain advantages of the proposed method over the Sørensen and Haugaard orcinol method.³⁵ In the tryptophane method absorption curves exhibit maxima with filter No. S-50 and errors due to humin formation from carbohydrate and the tryptophane occurring naturally in proteins are eliminated. Sørensen and Haugaard attempted to rule out humin discolorations in their orcinol method by using compensating blanks; but, the humin represents protein carbohydrate.

Absorption curves for pure sugar solutions differ from those of protein-sugar mixtures in both methods, and should therefore be standardized against carbohydrates added to protein. Either method allows qualitative differentiation of protein sugars by means of time curves depicting ratios between suitable filter readings, but the necessary reference data must be determined in protein-sugar mixtures.

The Accuracy of Routine Carotene Determinations As a Measure of Vitamin A Potency. BY LEO A. SHINN, EDWARD A. KANE, HERBERT G. WISEMAN, AND C. A. CARY. *From the Division of Nutrition and Physiology, Bureau of Dairy Industry, United States Department of Agriculture, Beltsville*

³⁵ Sørensen, M., and Haugaard, G., *Biochem. Z.*, **260**, 247 (1933).

It is well known that the Willstätter and Stoll procedure for preparing carotene does not yield a pure product. Colored impurities are present. The question arises as to the accuracy of routine carotene analyses based on this procedure.

In previous work carotene extracts, prepared in this way from alfalfa hay, were found to possess hardly significantly less (20 per cent) vitamin A activity than amounts of β -carotene that were equivalent in absorption at wave-length 436 $m\mu$. The spectral absorption of numerous extracts of this sort has now been determined. With freshly cut plant materials (alfalfa, blue-grass, corn plant) and leaf meals from artificially dried alfalfa, it resembles β -carotene closely; but with market hays and all silages that were tested, the relation between the absorptions at wave-lengths 430, 450, and 480 $m\mu$ differs from those of the carotenes or xanthophylls that might be present. This discrepancy is greater with the hays of poorer quality.

These extracts have also been filtered through Tswett columns of MgO (Strain). This treatment is reported not to change the spectral absorption of β -carotene materially through isomerization; and we have filtered it and mixtures of it with xanthophyll without oxidizing the carotene. The pigments in the carotene extracts have generally, however, been altered by this procedure; and it is difficult to determine with certainty the spectral absorption of the impurities in them. Based on the data now at hand, it appears likely that frequently 20 to 40 per cent of absorption of these extracts at wave-length 450 $m\mu$ is not due to carotene.

The Phospholipids of the Intestinal Mucosa during Fat Absorption.

BY ROBERT GORDON SINCLAIR. *From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York*

The object of this investigation has been to study further (1) the rôle of the phospholipids of the intestinal mucosa in fat resynthesis and (2) the factors which govern the composition of the phospholipids. Fats or esters containing chiefly a single fatty acid are fed to cats. The changes in the composition of the mucosa phospholipids are followed by determining the relative proportions and iodine number of solid and liquid fatty acids.

When elaidic acid is fed, the ratio of solid to liquid acids changes

from a control value of about 35:65 to as high as 60:40, and the elaidic acid makes up 80 per cent of the solid acids. About 65 per cent of the saturated acids and about 40 per cent of the unsaturated acids have been replaced by elaidic acid. This fact harmonizes well with the proposed intermediary rôle of the phospholipids in fat resynthesis.

However, when oleic acid is fed, there is no significant change in the ratio of solid to liquid acids, though there is a drop in the iodine number of the unsaturated acids which indicates that oleic acid has replaced some of the other unsaturated acids. The apparent failure of oleic acid to displace saturated acids from the phospholipids of the mucosa is, at the time of writing, not understood. It is hoped that the contemplated extension of the work will provide an answer.

The Activity Coefficients of Some Amino Acids. BY ELIZABETH R. B. SMITH AND PAUL K. SMITH. *From the Laboratories of Physiology and of Pharmacology and Toxicology, Yale University School of Medicine, New Haven*

The thermodynamic activity coefficients, γ , of certain amino acids, in aqueous solution at 25°, have been determined by equilibration of solutions through the vapor phase in a vacuum desiccator³⁶ with sucrose as the reference standard. Curves of γ as a function of molal concentration have widely different initial slopes for different amino acids (from -0.20 for glycine to +0.15 for valine). The effect of additional CH₂ groups in a straight chain is a definite increase in γ , as shown by comparing alanine and α -amino-*n*-butyric acid with glycine; this effect was greatest between glycine³⁷ and alanine, for which γ is 0.854 and 1.023, respectively, at 1.0 M. A comparison of two branched chain compounds, valine and α -aminoisobutyric acid, showed a similar but smaller effect. The curve for the latter compound was steeper than that for the normal isomer, indicating that a branched chain produces a more rapid change of γ with concentration than does a straight chain. Substitution of an additional polar group in alanine depresses the value of γ to an extent comparable with the

³⁶ Robinson, R. A., and Sinclair, D. A., *J. Am. Chem. Soc.*, **56**, 1830 (1934).

³⁷ Smith, E. R. B., and Smith, P. K., *J. Biol. Chem.*, **117**, 209 (1937).

removal of CH_2 from the chain, the values at 0.5 M being 1.012 for alanine, 0.910 for serine, and 0.915 for glycine.

The results as a whole indicate that size and shape are primary factors in determining the values of the activity coefficients of the α -amino acids.

Hemoglobin Regeneration at Different Levels of Iron Intake in Rats Made Anemic in the Presence of Copper. BY MARGARET CAMMACK SMITH AND LOUISE OTIS. *From the Nutrition Laboratory, Agricultural Experiment Station, the University of Arizona, Tucson*

Rats were made anemic by the Elvehjem-Kemmerer technique, except that copper was added to the whole milk diet. When the hemoglobin concentration reached 4 gm. per 100 cc. of blood, iron as ferric chloride supplemented with copper and manganese was fed at nine levels (0.014 to 0.5 mg. daily) for a 6 weeks test period. The resulting hemoglobin gains were strikingly lower than those previously reported at the same levels of iron intake in animals which had not received added copper in the anemia preparation period.

In the light of present knowledge, it appears that the added copper in the anemia production period made more complete utilization of iron reserves possible, so that animals thus rendered anemic had no stores of iron. To the contrary, copper reserves in the rats which received no copper other than that in the milk were exhausted before all of the iron reserve had been converted into hemoglobin. Therefore, upon the subsequent feeding of copper with iron, these iron reserves became available for hemoglobin formation. The resulting gains in hemoglobin were therefore not a true measure of the response to iron, but were abnormally large owing to this effect of copper in conversion of the body reserves of iron into hemoglobin. Unless rats are made anemic in the presence of sufficient copper for the complete utilization of iron reserves, their use for measurement of available iron in foods will give erroneous results, because they are influenced not only by the iron but also by the copper in the food under test.

Microdetermination of Collagen. BY HOWARD C. SPENCER. *From the Department of Biochemistry, University of Nebraska College of Medicine, Omaha*

In connection with our studies on dystrophic muscles it seemed desirable to find a chemical method for determining the degree and extent of the dystrophic change. Owing to the progressive accumulation of fibrous tissue the determination of the collagen content should furnish the necessary basis for a quantitative chemical evaluation of the dystrophy. The method for the collagen N determination as finally developed is as follows: Muscle freed from adhering tissue is minced, weighed, and dried by acetone. Drying to constant weight is completed in a vacuum desiccator. The material is powdered. A 200 mg. sample is weighed into a centrifuge tube, suspended in 4 cc. of water, and autoclaved for 2 hours at 15 to 20 pounds. After centrifuging, the liquid is transferred to a large centrifuge tube, and the residue is washed three times with small amounts of hot water, which is also added to the large centrifuge tube. The solution containing all the gelatin is acidified with 5 drops of HNO_3 , treated with 5 cc. of 5 per cent tannic acid, and left in the ice box for 1 hour. The gelatin is precipitated quantitatively. The precipitate is centrifuged off, washed with 5 to 10 cc. of tannic acid, dissolved in 1 cc. of 2 N NaOH, and transferred to a digesting flask. After neutralization with 2 cc. of 2 N H_2SO_4 the material is digested with 2 cc. of H_2SO_4 (1:1) and H_2O_2 , made up to a 100 cc. volume, and the N determination is carried out by nesslerizing aliquots of the solution. This procedure gives theoretical results on isoelectric gelatin.

The Effect of Bile Salts on the Enzymatic Synthesis and Hydrolysis of Cholesterol Esters in Blood Serum. BY WARREN M. SPERRY AND V. A. STOYANOFF. *From the Chemical Laboratory, Babies Hospital, and the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York*

This investigation extends recent studies²⁸ which showed that increasing amounts of sodium glycocholate inhibit increasingly the esterification of free cholesterol in incubated human or dog serum. With large concentrations cholesterol esters are completely hydrolyzed in dog serum, but in human serum there is neither esterification nor splitting. Taurocholate has essentially

²⁸ Sperry, W. M., and Stoyanoff, V. A., *J. Biol. Chem.*, **117**, 525 (1937).

the same effect as glycocholate. Cholate inhibits esterification in both species but causes hydrolysis of cholesterol esters in dog serum to only a small degree with molar concentrations equivalent to those of glycocholate and taurocholate which promote complete splitting. Desoxycholate inhibits esterification completely in all concentrations except the smallest studied and does not promote hydrolysis in dog serum. Monkey serum reacts toward taurocholate as does human serum. With taurocholate small amounts of untreated dog serum catalyze the complete splitting of cholesterol esters in human or dog serum inactivated by heating at 55-60° for 1 hour. Several preparations from serum have been made in an attempt to concentrate the enzyme or enzymes. Some of these added in small amount to untreated or heat-inactivated human or dog serum promote complete splitting of cholesterol esters in the presence of taurocholate, but they inhibit wholly or in part the normal esterification (without bile salt) in both species. No preparation able to catalyze esterification in heat-inactivated serum has been obtained.

The Dependence of the Extent of Synthesis of *p*-Bromophenylmercapturic Acid in Dogs on Body Weight. BY JAKOB A. STEKOL. *From the Department of Chemistry, Fordham University, New York*

Our earlier work has indicated that dietary sulfur is probably not the immediate source of the sulfur which is used in the detoxication of bromobenzene. It appeared to us of interest to investigate the dependence of the extent of the synthesis of *p*-bromophenylmercapturic acid on the body weight of the animal.

Litter mate female pups, 6 weeks old, were fed a synthetic diet of constant composition which was shown to induce growth in dogs to full maturity, and the urine was collected every 24 hours. Food intake was controlled and adjusted with growth as described previously. A 0.5 gm. single dose of bromobenzene was fed each month to each pup. The extent of the synthesis of the mercapturic acid was thus correlated with sulfur intake and body weight for a period of 10 months.

At the age of 6 weeks, the pups, weighing approximately 1.6 kilos each, excreted 20 to 28 per cent of the bromobenzene fed as mercapturic acid. With growth, the detoxication increased,

reaching 70 to 75 per cent efficiency at the age of 10 months. No proportionality of the extent of the mercapturic acid synthesis with sulfur intake was found. Much more consistent values were obtained when the extent of the synthesis of the mercapturic acid was correlated with the body weight of the dog.

Studies on the Constitution of Insulin. Properties of —SH— Insulin and the Reaction of —S—S— Insulin with Ketene. By KURT G. STERN AND ABRAHAM WHITE. *From the Laboratory of Physiological Chemistry, Yale University, New Haven*

Reduced insulin preparations²² have been further studied by physical and chemical methods. Compared with the original (—S—S—) insulin, the reduced products have a practically identical content of tyrosine, free amino groups, and total sulfur. The molecular size, the isoelectric point, the viscosity, and the ultraviolet absorption spectrum are also found to be unchanged. These findings support the conclusion²² that the only alteration produced by the reaction of insulin with thioglycolic acid is a reduction of a small number of dithio linkages in the hormone to sulfhydryl groups.

The time course of acetylation of —S—S— insulin with ketene has been studied at various temperatures. It has been possible to prepare an acetyl derivative in which all of the free amino groups but no appreciable number of phenolic hydroxyl groups of insulin have been acetylated. This product may be obtained in crystalline form. Preliminary bioassay indicates that products of this type have a physiological activity comparable to that of the original insulin powder (20 to 22 units per mg.). Preparations are being studied in which not only free amino but also other groups, e.g. tyrosine, have been blocked by acetyl radicals.

A Study of "Ascorbic Acid Oxidase" in Relation to Copper. By ELMER STOTZ, CARTER J. HARRER, AND C. G. KING. *From the Department of Chemistry, University of Pittsburgh, Pittsburgh*
Considerable evidence has accumulated to suggest that purified squash "oxidase" and Hopkin's cauliflower preparation oxidize ascorbic acid chiefly by virtue of their copper content. The cata-

²² Stern, K. G., and White, A., *J. Biol. Chem.*, **117**, 95 (1937).

lytic properties of copper solutions are influenced greatly by protein; hence copper + albumin and copper + gelatin have been compared with the "enzymes."

The amount of copper in the preparations is sufficient, if present in inorganic form, to produce a velocity of oxidation much greater than that produced by the "enzyme." The activity of copper + albumin is governed by the amount of both copper and albumin, there being a progressive decrease in activity with increased amounts of albumin.

Seven copper inhibitors, including diethyl dithio carbamate, produced inhibitions of the oxidation ranging from 65 to 95 per cent, with both the "enzymes" and copper, with or without albumin and gelatin. An apparent lower activity of the inhibitors on cauliflower "oxidase" was comparable to copper + albumin of equal protein content.

The decrease of activity caused by heating the "enzyme" also occurred with copper + albumin. It is suggested that the effect of heating is due to removal of copper with coagulated protein. The larger part of the copper originally present was found in the coagulum (inactive).

Copper + albumin (like the "enzyme") exhibits an optimum pH and inactivation by acid.

Partial inactivation by trypsin is attributed to the observed greater copper-inhibiting action of the products of proteolysis.

The Cystine Content of Insulin. BY M. X. SULLIVAN AND W. C.

Hess. *From the Chemo-Medical Research Institute, Georgetown University, Washington*

In early work with insulin, both amorphous and crystalline, the cystine found in the 20 per cent HCl hydrolysate by three different methods was, Sullivan 8.5, Okuda 8.7, Folin-Marenzi 8.6. The total sulfur in the hydrolysate indicated approximately 9 per cent and showed sulfur was lost in the hydrolyzing. This volatile S was captured in later work. By means of the cystine determinations and the sulfur evolved during hydrolysis, all the S of the insulin was explained. The hydrolysis was repeated as recommended by du Vigneaud (private communication) by equal volumes of 35 per cent HCl and concentrated formic acid, evaporating to dryness, and making to volume in 0.1 N HCl. The

cystine found was, Sullivan 11.07; Okuda 11.65; Folin-Marenski 11.53, or 92.3; 97.1 and 96.1 per cent of the total sulfur. All the figures are uncorrected for moisture and ash.

Can Crystalline Urease Be Made to Function As Arginase? BY JAMES B. SUMNER AND ALEXANDER L. DOUNCE. *From the Department of Physiology and Biochemistry, Cornell University Medical College, Ithaca*

Hellerman and Perkins⁴⁶ have made the claim that when crystalline urease is treated with cobaltous chloride it is able significantly to hydrolyze arginine into urea and ornithine. The authors find that the jack bean contains a rather large amount of the enzyme arginase and that this is greatly activated by cobaltous chloride as well as by various other metallic salts. Crystalline urease does contain some arginase, but this diminishes upon recrystallizing once, and is practically absent after two recrystallizations. On the other hand, it is an easy matter to obtain jack bean extracts of powerful arginase action but entirely free from urease activity provided one adds an excess of iodine to jack bean preparations, followed by a slight quantity of thiosulfate.

The Rôle of Vitamin B₁ in Cardiovascular Diseases. BY BARNETT SURE AND W. A. JONES. *From the Department of Agricultural Chemistry, University of Arkansas, and the United States Veteran's Hospital, Fayetteville*

Because of the striking resemblance of the symptoms of cardiac disease and accentuated cases of vitamin B₁ deficiency, it is not unlikely that certain cardiac diseases may be due to the cumulative effects of vitamin B₁ deficiency extending over a period of a number of years, as it is a known fact that the general American diet is deficient in vitamin B₁. An opportunity presented itself for a preliminary study of this kind at the United States Veteran's Hospital at Fayetteville. It was the aim to have a diet that was easily assimilated, yet furnishing sufficient food energy and being high in vitamin B₁. Thirty patients were under this regimen. Eighteen patients, whose condition was more severe, received

⁴⁶ Hellerman, L., and Perkins, M. E., *J. Biol. Chem.*, **112**, 175 (1935-36).

a daily supplement of a vitamin B₁ concentrate which added 1000 units to the diet. The daily allowance was 1500 to 2000 Sherman units. In addition each patient received two glasses of orange juice daily. The average length of hospitalization was 114 days. From the improvement in respiration, pulse rate, and blood pressure, and the ability of the patients to indulge in exercise following the dietary treatment during the period of study, it is concluded that the diet abundant in vitamin B₁ exerted a very favorable influence on the cases of heart disease under observation.

The majority of these patients showed inefficiency in the utilization of vitamin C, as evidenced from daily determinations of urinary excretion during 10 day periods. Such findings would indicate greater requirements of this vitamin in cardiac diseases.

The Nature of a Gastric Ulcer-Producing Substance Isolated from Muscle. BY SHIRO TASHIRO. *From the Biochemical Laboratory, University of Cincinnati, Cincinnati*

The author has suggested that the muscle "antigen" of Hinton used for the syphilis test might contain a bile salt, because it has the power to produce ulceration in the stomach of a guinea pig, gives a strong Pettenkofer reaction, and because he has prepared (with Miss Vack) synthetic "antigens" containing conjugated bile salts as the main constituent. An attempt to isolate bile acids from dogfish muscle by White's method was, however, not successful; no desoxycholic or cholic acid could be obtained and the Mg salt was not active. Instead, there was obtained a crystalline, exceedingly hygroscopic substance, 3 times as active in gastric ulcer-producing power as conjugated bile acids, and which has the additional power to make the guinea pig shed "milky" tears and the rat bloody tears. Pending further chemical identification, the name of dacryorrhetin is given to this substance. An intracardial injection of 0.2 mg. per 100 gm. of body weight of dacryorrhetin causes the rat to shed bloody tears instantaneously, and an intraperitoneal injection of 3 mg. produces a similar reaction in the rat in 2 minutes, milky tears in the guinea pig in 2 to 3 minutes, and ulceration of the stomach in 3 hours. Unlike the ulcer-producing action of bile salts, that of dacryorrhetin is not antagonized by lecithin.

The Alleged Formation of a Sulfenic Acid by the Oxidation of Cysteine with Dithioformamidine. BY GERRIT TOENNIES.

From the Lankenau Hospital Research Institute, Philadelphia

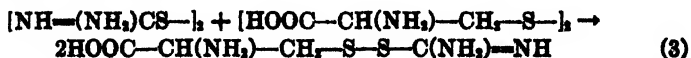
In 1933 Pirie⁴¹ studied polarimetrically the reaction between cysteine and dithioformamidine, the disulfide corresponding to thiourea. The observation that when the hydrochlorides of the two components reacted in the ratio of 2 cysteine and 1 dithioformamidine, cystine was formed, while, when the ratio was 1:1 or less, a constant polarimetric value equal to less than one-half of that corresponding to cystine resulted, together with observations on the resulting solution were interpreted as indicating oxidation of cysteine to the corresponding sulfenic acid, according to the equation



This interpretation was found to be erroneous. The reaction is



The same product is formed from cystine and dithioformamidine, according to the equation



This reaction is slow compared with reaction (2) and is catalyzed by thiourea.

The assymetric disulfide resulting according to reaction (2) or (3) has been isolated as the hydrochloride and found to possess the expected properties. It is relatively stable in acid solution, while in neutral solution it appears to hydrolyze into thiourea and the sulfenic acid corresponding to cysteine, which undergoes further dismutation.

The Effect of Calciferol on the Serum Calcium Level in the Nephrectomized and in the Thyroparathyroidectomized-Nephrectomized Rat. BY WILBUR R. TWEEDY, E. W. Mc-

⁴¹ Pirie, N. W., *Biochem. J.*, **27**, 1181 (1933).

NAMARA, R. D. TEMPLETON, AND MARY C. PATRAS. *From the Departments of Physiological Chemistry and Physiology, Loyola University School of Medicine, Chicago*

The average serum calcium value in fourteen rats was 10.53 mg. per cent 48 hours after bilateral nephrectomy. In thirteen other animals, which were bilaterally nephrectomized and injected subcutaneously with 34.5 mg. (1,380,000 international units) of calciferol in divided doses, the minimum, maximum, and average serum calcium values after 48 hours were 13.20, 19.80, and 16.31 mg. per cent, respectively.

Eight rats, which were thyroparathyroidectomized and immediately bilaterally nephrectomized, were injected with 11.5 to 34.5 mg. of calciferol. The serum calcium was elevated, or fell markedly. In nineteen other animals, which were bilaterally nephrectomized 21 to 31 days after parathyroidectomy, or thyroparathyroidectomy, and similarly treated with calciferol, the minimum, maximum, and average serum calcium values observed after 48 hours were 5.02, 10.76, and 6.79 mg. per cent.

Oscillographic Study of the Cytochromes during Muscular Contraction. BY FRANK URBAN AND HUBERT B. PEUGNET. *From the Laboratory of Biological Chemistry and the Department of Physiology, Washington University School of Medicine, St. Louis*

Directly stimulated gastrocnemii of *Rana pipiens*, firmly held between parallel glass plates, were successively illuminated during contraction with different wave bands: 700 to 625 $m\mu$ (non-specific); 610 to 595 $m\mu$ (cytochrome *a*); 568 to 555 $m\mu$ (cytochrome *b*); 558 to 542 $m\mu$ (cytochrome *c*), respectively. The changes in transmission during contraction were recorded, by a photocell and a condenser-coupled amplifier, with a cathode ray oscillograph. The differences between the non-specific and the cytochrome oscillograms show activity of cytochrome *c* in the early phases of the muscular contraction. Cytochrome *a* is probably active during the relaxation phase. The activity of cytochrome *b* is being investigated. Since the observations were taken on the first twitches of rested, freshly excised muscles only, they may be called aerobic. The activity of the cytochromes during contraction is thought to be intimately related to the contractile process.

Precursors of Taurocholic Acid. BY ROBERT W. VIRTUE AND MILDRED E. DOSTER-VIRTUE. *From the Department of Biochemistry, Louisiana State University Medical Center, New Orleans*

This study was designed to obtain information regarding the origin of taurine. Fasting, bile fistula dogs weighing about 12 kilos were given 2.8 gm. of cholic acid daily. Feeding an equivalent amount of cystine (0.8 gm.) or methionine (1.0 gm.) in addition was followed by an increased output of taurocholic acid in the bile. No such increase was observed after administration of alanine with the cholic acid. In one experiment in which homocystine (0.9 gm.) was fed with the cholic acid no increased production of taurocholic acid was observed. In another experiment with homocystine the level of taurocholic acid excretion was somewhat elevated for 3 days, but the data are too inconsistent to suggest that homocystine caused extra production of taurine. Taurocholic acid was determined by a method essentially that of Foster and Hooper.

In other experiments on dogs we observed that material giving disulfide reactions was excreted in the urine after monobromobenzene was fed. Negative Rossouw-Wilken-Jorden tests indicated that the material was not cystine.

Crystalline Ficin. BY A. WALTJ. *From the Research Laboratories of Merck and Company, Inc., Rahway, New Jersey*

The crystalline proteolytic enzyme isolated from a *Ficus* latex obtained from Central America is a true catheptidase or papainase. Like the latter, it can be inactivated by oxidizing agents such as hydrogen peroxide, iodine, oxygen, cystine, and phenylhydrazine. On the other hand, the inactivated form can be reactivated with cysteine. The optimum of the action of the enzyme for gelatin is pH 5.

The enzyme is characterized by a very high sulfur content, reaching a value of 1.6 per cent, a value higher than that reported for any other crystalline enzyme of protein nature. It was established that sulfur is present in the form of —SH groups. This was demonstrated by the modified nitroprusside reaction. If the test is carried out by the conventional method, it may fail to reveal the presence of an —SH group.

Basal Metabolism, Preformed, and Total Creatinine Nitrogen in Twenty-Four Boys and Forty-Six Girls between One Month and Fifteen Years of Age. BY CHI CHE WANG. *From the Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati, Cincinnati*

The averages of 6 day periods of basal metabolism, preformed and total creatinine nitrogen, and the ratios of basal metabolism and the two forms of creatinine nitrogen were correlated with age, height, weight, and surface area. Basal metabolism was also correlated with preformed and total creatinine nitrogen. Furthermore, the values were grouped and averaged according to age, weight, height, and surface area.

The results demonstrated the following. (1) All the correlation coefficients were positive and high, ranging from ± 0.981 to ± 0.879 . (2) Unexpectedly, preformed creatinine nitrogen correlated most closely with weight and least with basal metabolism. (3) Basal metabolism correlated more closely with height than with age, weight, or surface area. (4) Both basal metabolism and preformed creatinine increased with weight, but the former augmented more slowly than the latter, as was evidenced by the gradual decline of metabolism per kilo and the steady rise of the ratio between creatinine and basal metabolism. (5) Beginning with children from 9 years, or 26 kilos, or 141 cm., or 0.901 sq.m. upward, the rate of increase of preformed creatinine per day gradually declined, resulting in a per kilo value approaching constancy. (6) The total creatinine nitrogen and its ratio to basal metabolism also augmented with growth, but values were less consistent, probably due to the influence of diet.

A Reaction of Ascorbic Acid with Formaldehyde. BY EDWARD S. WEST AND LUMAN F. NEY. *From the Department of Biochemistry, University of Oregon Medical School, Portland*

When formaldehyde is added to ascorbic acid, the latter quickly loses its power to reduce 2,6-dichlorophenol indophenol. The compound formed is unstable and the formaldehyde may be removed by treatment with dimedon. By the reaction of formaldehyde with ascorbic acid in the presence of calcium carbonate, a thick, sweet, almost colorless syrup is formed, which reduces alkaline copper solutions but not the indophenol reagent. Dime-

don does not precipitate formaldehyde from solutions of the compound. The syrup gives $[\alpha]_D^{25} = -12.8^\circ$ to -14° in water, and a molecular weight of 234 (freezing point), corresponding to the addition of two CH_2O . Acetylation indicates four hydroxyl groups.

If formaldehyde is an intermediate in the photosynthetic process in plants, its combination with ascorbic acid may represent an important stage in the process. The studies are being continued.

The Effect of Vitamin B₁ on the Respiratory Quotient. BY DOROTHY V. WHIPPLE AND CHARLES F. CHURCH. *From the Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia*

Previous work from this laboratory has indicated that the tissues of vitamin B₁-deficient rats contained less fat than the tissues of control rats receiving an isocaloric intake and an adequate amount of the vitamin. In the present investigation respiratory quotients after subcutaneous injections of 1 gm. of glucose were determined. Vitamin B₁-deficient rats showed respiratory quotients below 1, while their isocaloric controls receiving the vitamin showed quotients well above unity. (The average of seventeen determinations on eight rats taken during the progress of vitamin B₁ depletion was 0.85. The average of seventeen determinations on nine control rats receiving an adequate amount of vitamin B₁, made on the same days and under the same conditions as the above, was 1.15.)

Similarly, crystalline vitamin B₁ raised the respiratory quotients of animals showing severe symptoms of beriberi. The average of eight determinations on seven rats showing severe symptoms was 0.79. The average of seven determinations on these same seven rats immediately after the subsequent injection of 0.1 mg. of crystalline vitamin B₁ was 1.48.

These observations are in harmony with the hypothesis that vitamin B₁ is an essential metabolic constituent for the synthesis of fat in the animal body.

Some Products of the Partial Hydrolysis of Crystalline Edestin by Pepsin. BY ABRAHAM WHITE. *From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*

Pepsin has been allowed to act upon crystalline edestin, in hydrochloric acid solution, until tests for unchanged globulin are negative. At this point, corresponding to the liberation of approximately 20 per cent of the total amino nitrogen which could be obtained by complete hydrolysis of the protein with strong acid, enzymatic action was interrupted. Digests prepared in this way have been fractionated with the aid of inorganic salts. Each of the purified fractions, as well as the original protein, has been analyzed for total nitrogen, amino nitrogen, total sulfur, cystine, tyrosine, tryptophane, arginine, histidine, and lysine and some interesting differences have been observed.

The Cholesterol Content of Dystrophic Rabbits. BY VIOLET M.

WILDER. *From the Department of Biochemistry, College of Medicine, University of Nebraska, Omaha*

It has been found in our laboratory that the most outstanding chemical change in rabbits affected with nutritional muscular dystrophy is the very great increase in cholesterol content of the skeletal muscles and of the blood. Some organs (liver, lung, and spleen) showed a small, variable decrease in cholesterol. To decide whether we were dealing primarily with a redistribution of preformed cholesterol or with an actual synthesis of cholesterol in the dystrophic animals we carried out the determination on the entire animal, both normal and dystrophic. The animals, killed by a blow on the head, were depilated with a solution of Na_2S and freed from the intestinal contents. The carcass was weighed and autoclaved 2 hours at 35 pounds pressure. The material was then ground and mixed to a homogeneous mass. The finely ground mush was partially evaporated; samples were taken from several localities by means of a large sized cork borer and extracted several times with alcohol-ether mixture (3:1) as usual. The cholesterol was determined colorimetrically.

Both male and female rabbits were used (three of each), and four animals were analyzed when they became definitely dystrophic.

In the controls we found 145 to 163 mg. and in the dystrophic animals 234 to 554 mg. per 100 gm. of live weight.

The increase of 50 to 360 per cent in the cholesterol content of the entire dystrophic rabbits indicates an actual synthesis of the substance in the development of the disease.

The Formation of Oxalic Acid. BY FRANK H. WILEY, DOROTHY S. BERGEN, AND FRANK R. BLOOD. *From the Haskell Laboratory of Industrial Toxicology, Wilmington, Delaware*

In an attempt to elucidate the mechanism of the formation of oxalic acid in the animal organism, we have studied the excretion of this substance in the urine of dogs and rabbits following the subcutaneous injections of approximately equivalent quantities of ethylene glycol, dioxane, ethylene glycol monoacetate, ethanol amine, dihydroxy ethyl ether, and the monomethyl and the diethyl ethers of ethylene glycol.

Of the material studied, only ethylene glycol and its monoacetate gave an increased excretion of oxalic acid. These results, together with the results of Herkel and Koch, who found that glyoxal and glycolic acid increased the excretion of oxalic acid, indicate that the production of oxalic acid from any material depends upon its metabolic transformation into a 2-carbon compound in which both carbons are partially oxidized.

Displacement of Globin from Hemoglobin by Pyridine. BY EDWARD F. WILLIAMS, JR., AND DEMPSIE B. MORRISON. *From the Department of Chemistry, College of Medicine, University of Tennessee, Memphis*

The following equilibria have been studied at pH values less than 7.



To fresh oxyhemoglobin solutions, 2 to 11 mM in respect to heme, were added equal volumes of pyridine-pyridinium chloride solutions, 50 to 2000 mM, in respect to total pyridine, the ratio of pyridinium ion to total pyridine having been adjusted by means of hydrochloric acid. After standing, the pH of each mixture was measured and an aliquot diluted with acetone to 10 volumes to precipitate the proteins. The concentration of hemin in the 90 per cent acetone solution was determined spectrophotometrically. Concentrations were so adjusted that only a part of the total globin was displaced from the hemoglobin of the mixtures. The pH being known, the pyridinium ion concentration necessary

to displace 50 per cent of the globin was ascertained. 7.7 ± 0.6 mm of pyridinium ion per mm of original hemoglobin are required to bring about such half displacement. The degree of displacement is directly proportional to the concentration of pyridinium ion, but independent of pH except in so far as the latter governs pyridinium ion concentration. Addition of 1900 to 2000 mm pyridine immediately precipitated the hemoglobin and results in these mixtures were abnormal. The range of 1000 to 1900 mm pyridine is being investigated. In the absence of pyridine, but with addition of hydrochloric acid alone, 50 per cent of the heme was removed from the hemoglobin at $\text{pH } 4.05 \pm 0.11$.

Influence of Bromide and Chloride in the Diet on the Amount of Bromine in the Body. BY PHILIP S. WINNEK AND ARTHUR H. SMITH. *From the Laboratory of Physiological Chemistry, Yale University School of Medicine, New Haven*

The growth of young rats on a synthetic diet containing less than 0.5 part of bromine per million and having a Br:Cl ratio of 0.00043 was compared to the growth of litter mates receiving the same diet with potassium bromide added, so that the food contained 25.0 parts of bromine per million and had a Br:Cl ratio of 0.0215. No significant differences were found in growth and general appearance of the two groups of rats. The amount of bromine in the blood and certain tissues of the animals was determined and compared with that of animals from the stock colony which received a diet composed of Calf Meal containing 16.5 to 20.0 parts of bromine per million and having a Br:Cl ratio of approximately 0.001. The amount of bromine in the blood of the animals on the synthetic low bromine diet was found to be from 0.055 to 0.280 mg. per cent, on the synthetic diet with potassium bromide added, 7.20 to 9.90 mg. per cent, and on the stock diet 1.35 to 2.39 mg. per cent. Similar large differences were found in the tissues analyzed. Since the animals on the stock diet received nearly as much bromine in their food but contained much less bromine in their bodies than the animals on the synthetic diet with added potassium bromide, it appears that the ratio of Br:Cl as well as the absolute amount of bromine in the diet influences the amount of bromine in the body.

